Aggrecan Degradation in Health and Disease

Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy, University of Wales

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Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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My heartfelt thanks to my partner Rob, who has managed to keep me almost sane over the last 3 years and without whom this would not have been possible.

Contents

Abbreviations

α	Anti
A1-A4	Associative Fractions 1-4
A1D1	Associative Fraction 1 Dissociative Fractions 1
A1D1-A1D4	Associative Fraction 1 Dissociative Fractions 1-4
ABC Kit	Avadin Biotin Complex kit
ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ANOVA	Analysis Of Variance
AP-1	Activating Protein-1
APES	Amino Propyl Triethoxysilane
BCIP	5-Bromo-4-Chloro-3-Indoyl-Phosphate
β-El	Alkaline β Elimination
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumen
C-4-S	Chondroitin-4-Sulphate
C-6-S	Chondroitin-6-Sulphate
CA-MMP	Cysteine Array Matrix Metalloproteinase
CD	Cluster of Differentiation
CHX	Cycloheximide
CILP	Cartilage Intermediate Layer Protein
COL	Helical Collagenous Domain
COMP	Cartilage Oligomeric Matrix Protein
CPC	Cetylpyridinium Chloride
CS	Chondroitin Sulphate
CUB	Complement Cla/Clr sea urchin Uegf protein, Bone Morphogenetic Protein-1
DAB	Diaminobenzine

DMEM	Dulbecco's Modified Eagles Medium
DMMB	Dimethyl Methylene Blue
DMSO	Dimethyl Sulphonyl Oxide
DPX	P-xylene-bis / N-pyridium Bromide
DS	Dermatan Sulphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diaminetera Acetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
FACIT	Fibril Associated Collagen with an Interrupted Triple Helix
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
G1-3	Globular Domains 1-3
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	Galactosamine
Glc	Glucose
GlcNAc	Glucosamine
Glu	Glutamine
GPI	GlycoPhosphatidyl Inositol
Gx	Guanidine Extracts
HA	Hyaluronic Acid (Hyaluronan)
HCl	Hydrochloric Acid
HME	Human Macrophage Metalloelastase
HRP	Horse Radish Peroxidase
HS	Heparan Sulphate

Hspg2	Heparan Sulphate Proteoglycan 2
Ig	Immunoglobulin
IGD	Interglobular Domain
IGF	Insulin-like Growth Factor
IL	Interleukin
K_I	Inhibition constant
KS	Keratan Sulphate
LP	Link Protein
LRR	Leucine Rich Repeat
M'Ab	Monoclonal Antibody
MB	Metalloproteinase Clan B
METH	Metalloproteinase with Thrombospondin Domains
MME	Macrophage Metalloelastase
MMP	Matrix Metalloproteinase
MT-MMP	Membrane Type Matrix Metalloproteinase
MW	Molecular Weight
NBT	Nitroblue Tetrazolium
NC	Non-Collagenous Domain
N-CAM	Neural Cell Adhesion Molecule
N-TIMP-3	Recombinant protein of Amino-terminal region of TIMP-3
OA	Osteoarthritis
PACE	Paired basic Amino acid Cleaving Enzyme
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween 20
PCR	Polymerase Chain Reaction
PG-Lb	Proteoglycan Leucine rich
pH	per Hydrogen
Phos C	Phosphatin C

PLAC	Protease Lacunin
PMNL	Polymorphonuclear Leucocyte
PMSF	Phenyl Methyl Sulphonyl Fluoride
PRELP	Protein Arginine-rich and leucine rich repeat protein
PRG-4	Proteoglycan-4
PTR	Proteoglycan Tandem Repeat
PUMP	Putative Metalloproteinase
RASI	Rheumatoid Arthritis Synovium Inflamed-1
RNA	Ribose Nucleic Acid
RPM	Revolutions Per Minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SLRP	Small Leucine rich Repeat Proteoglycan
TA	Tenascin Assembly Domain
TACE	Tumour Necrosis Factor α Converting Enzyme
TBE	H-ras oncogene transformed human bronchial epithelial cells
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinase
TNFα	Tumour Necrosis Factor alpha
TSA	Tris Saline Azide
TSP	Thrombospondin
UDP	Uridine Diphosphate
v/v	volume per volume
vWFA	von Willebrand Factor A Domain
w/v	weight per volume

Quantities

p	pico	1×10^{-12}
n	nano	1×10^{-9}
µ	micro	1×10^{-6}
m	milli	1×10^{-3}
k	Kilo	1×10^{3}
%	Percentage	
hrs	Hours	
°C	Degrees Celsius	
g	grams	
l	litres	
M	Molar	
D	Daltons	

Abstract

The main aggrecan catabolite, found in samples of synovial fluid from patients with arthritis, and released from cartilage explant cultures exposed to IL-1, both have the amino-terminal amino acid sequence ^{374}ARGSV... (human sequence enumeration) corresponding to cleavage at the 'aggrecanase site' within the IGD of aggrecan (Sandy *et al.*, 1992, and Lohmander *et al.*, 1993). Loss of aggrecan is a primary event in the destruction of cartilage in arthritic disease. The Glu^{373}-Ala^{374} bond ('aggrecanase site') within the IGD of the aggrecan core protein is cleaved by members of the ADAMTS family, including ADAMTS-4 and -5 (Tortorella *et al.*, 1999, Abbaszade *et al.*, 1999, and Sandy *et al.*, 2000).

In this investigation the model system of chondrocyte-agarose cultures, pioneered by Aydelotte and Kuettner 1988, was used to study the degradation of aggrecan by ADAMTS-4 and -5 in cartilaginous extracellular matrices.

Low molecular weight co-migrating ~37kD ADAMTS-4 and -5 isoforms were detected in apparently increased amounts in IL-1α treated cultures compared to controls. These isoforms were bound by heparin and required *de novo* protein synthesis in the presence of IL-1α for their generation. As previously reported, *de novo* protein synthesis in the presence of IL-1α was also required for 'IGD aggrecanase activity' (Arner *et al.*, 1998). Heparin bound media fractions from IL-1α treated cultures possessed 'IGD aggrecanase activity' against exogenous aggrecan, which was inhibited by the amino-terminal region of TIMP-3 and was shown to be due to a 37kD isoform of ADAMTS-4. This implicated low molecular weight isoforms of ADAMTS-4 in the aggrecanolysis detected in the presence of IL-1α.

Carboxy-terminal truncation of furin cleaved ADAMTS-4 has previously been proposed as both an activation mechanism for the enzyme (Pratta *et al.*, 2003, Kashiwagi *et al.*, 2004, Gao *et al.*, 2002, Gao *et al.*, 2004 and Flannery *et al.*, 2002) and a means of deregulation of the enzyme's catalytic activity.(Gao *et al.*, 2002, Gao *et al.*, 2004, and Kashiwagi *et al.*, 2004). Therefore high molecular weight Furin cleaved ADAMTS-4 isoforms may be required to play normal physiological roles, whereas the low molecular weight forms are likely to be the enzyme isoforms involved in the destruction of aggrecan and other proteoglycans in articular cartilage during arthritis.

Chapter 1: Introduction

1.1 Articular Cartilage Morphology

To take the stress out of everyday movement the load bearing joints of the body are lined with articular cartilage. This aneural, avascular, alymphatic, hypocellular tissue acts to reduce friction and absorb impact. Articular cartilage is composed of a dense extracellular matrix controlled and secreted by the cells (chondrocytes) within it. The cells contribute less than 2% of the volume of mature adult articular cartilage; the remaining volume is occupied by collagens, proteoglycans and water. Water comprises 70% of the tissue's wet weight, collagens 20%, proteoglycans 7% and other proteins around 1% (Poole *et al.,* 2001, and Vertel 1995).

1.1.1 Articular Cartilage Chondrocytes

These are the only cell type present in articular cartilage and differ in both their morphology and metabolic activity between the various zones (see Figure 1.1). All contain an endoplasmic reticulum and Golgi apparatus necessary for matrix synthesis and secretion, but some may also contain intracytoplasmic filaments, lipid globules, glycogen and secretary vesicles. The cells are diffusely spread throughout the matrix allowing for no cell-cell contacts (Buckwalter and Hunziker 1999). Some chondrocytes have short processes or microvilli extending from their surface out into the matrix. These structures may sense mechanical changes in the matrix and relay this information to the cells (Buckwalter and Hunziker 1999) giving a feedback mechanism allowing adaptation in the composition of the matrix macromolecules in response to changes in the physical state of the matrix.

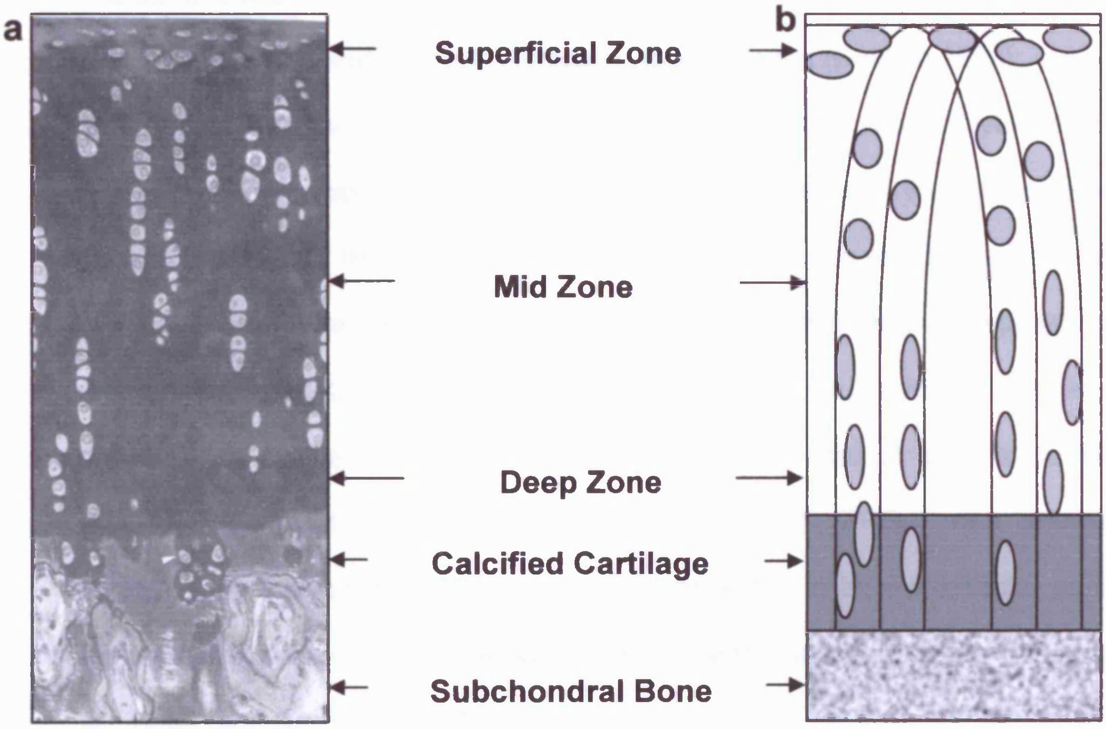

Figure 1.1 Zones of articular cartilage (a) Mature rabbit articular cartilage (Jimenez and Hunziker 1992) and Benninghoff Arcades of Collagen fibrils (Benninghoff 1925)

1.1.2 Morphological Zones

Mature articular cartilage may be divided into 4 overlapping zones (Figure 1.1a) running parallel to the cartilage surface. The arrangement of the cells within these zones is maintained by the collagen fibre organisation known as Benninghoff Arcades (Benninghoff 1925) (Figure 1.1b).

- Adjacent to the joint cavity is the superficial zone which can be divided into two layers:
  - The surface acellular layer is composed of a layer of amorphous material (Juvelin *et al.,* 1996) covering a sheet of fine fibrils rich in type I collagen (Duance 1983)
  - The deeper cellular layer contains flattened or ellipsoid chondrocytes and a high collagen concentration (Duance *et al.,* 1999). The balance of the proteoglycan types present differs from that seen in other areas of the cartilage with a lower concentration overall, and of that which is present a high concentration is composed of small proteoglycans. PRG-4, also known as Superficial Zone Protein (SZP) is a novel proteoglycan synthesised by superficial zone chondrocytes of articular cartilage and was first identified by Schumacher *et al.,* in 1994. PRG-4 was shown to be intracellularly located in superficial zone chondrocytes in bovine articular cartilage as well as being present in the fine layer of matrix at the articular surface (Schumacher *et al.,* 1999) (see Section 1.2.2.7).

  The dense collagen fibrils present in the superficial zone of articular cartilage lead to a greater tensile strength, as they lie parallel to the surface of the zone.
- The transitional, mid or intermediate zone lies below the superficial / surface zone and contains oval or rounded cells in groups of up to 2 or 3. Compared to the superficial zone this region has a higher proteoglycan concentration, lower collagen concentration and collagen fibrils of larger diameter. The collagen fibrils in this region are mainly type II collagen. Unique to this area of cartilage is Cartilage Intermediate Layer Protein (CILP) (see Section 1.2.3.3) (Lorenzo *et al.,* 1998a).
- The deep or radial zone contains large rounded cells in groups of 2, 4, 6 or 8 arranged in columns running perpendicular to the cartilage surface. Of all the zones this has the highest proteoglycan concentration, the lowest collagen concentration and the largest diameter

collagen fibrils (Buckwalter and Hunziker 1999). The collagen fibres pass into the so-called tidemark seen in decalcified cartilage between the deep and calcified zones.

- The calcified zone rests on the underlying subchondral bone. Here the matrix contains crystals of calcium salts and, as in the deep zone, the cells are found in groups of up to 8.

1.2 Articular Cartilage Extracellular Matrix Components

The articular cartilage matrix is composed of tissue fluid and the matrix macromolecules that give the tissue form and structure. The cartilage structural macromolecules, collagens, proteoglycans and non-collagenous proteins contribute 20-40% of the wet weight of the tissue (Buckwalter and Hunziker 1999). The three classes of macromolecules differ in their concentrations and their contributions to the mechanical properties of the tissue. Collagens contribute 60% of the dry weight of cartilage, proteoglycans 25-35% and non-collagenous proteins and glycoproteins 15-20% (Buckwalter and Hunziker 1999). The collagen fibril meshwork gives cartilage its form and tensile strength (Buckwalter and Mow 1992). Proteoglycans and non-collagenous proteins bind to the collagen meshwork or become mechanically trapped within it. The major proteoglycan of articular cartilage is aggrecan, which has numerous glycosaminoglycan chains attached to its core protein. These form a highly compressible structure when fully hydrated (see Section 1.2.2). Some non-collagenous proteins stabilize the matrix framework and others facilitate association of chondrocytes with the matrix (see Section 1.2.3).

1.2.1 Collagens

Collagens are the most abundant family of proteins in mammals. They form fibrous elements and are the most ubiquitous structures in most connective tissues. To qualify as a collagen a protein must contain a segment of at least 20 residues where every third residue is a glycine in the sequence Gly-X-Y (Kadler 1996). In each chain this sequence forms a left-handed helix and the chains wind around each other in a right-handed super triple helix. Twenty seven members of the collagen family have now been identified (Myllyharju and Kivirikko 2001, Fitzgerald and Bateman 2001, Gordon *et al.,* 2000, Gordon *et al.,* 2002, Hashimoto *et al.,* 2002, and Sato *et al.,* 2002), which are produced from over 30 genes, and they can be separated into classes according to their structure and functions as shown in Table 1.1 (Eyre 1991).

Table 1.1 Collagen family members and their tissue distribution (adapted from Eyre 1991, Eyre 2002, Boot-Handford *et al.*, 2003, and Koch *et al.*, 2003). Blue shading indicates minor articular cartilage collagens whilst red shading indicates major articular cartilage collagens.

COLLAGEN CLASS	COMMENTS	COLLAGEN TYPE	TISSUE DISTRIBUTION
Fibril Forming	These contain a large triple helical domain with around 330 Gly-X-Y repeats per chain and are synthesised as large precursors and assemble into cross striated fibrils, with each molecule being displaced ¼ of its length along the axis of the fibril relative to its nearest neighbour (Burgeson 1988)	**I**	skin, bone, tendon, intervertebral disc and **cartilage**
		II	vitreous, **cartilage**, intervertebral disc
		III	skin, blood vessels, **cartilage**, intervertebral disc
		V	skin, bone, tendon, intervertebral disc and **cartilage**
		XI	skin, bone, tendon, intervertebral disc and **cartilage**
		XXIV	developing cornea and bone
		XXVII	chondrocytes, developing stomach, lung, gonad, skin and tooth
Network Forming	Self assemble into networks and have longer non-collagenous domains than fibril forming collagens (Kadler *et al.*, 1996, and Prockop and Hulmes 1994). Monomers associate at their carboxy-termini to form dimers and at their amino-termini to form tetramers. The triple helical domains intertwine to form supercoiled structures (Hulmes 2001).	**IV**	basement membranes and stromal region of the cornea
		VIII	Descement's membrane and endothelial cells
		X	calcifying **cartilage**
Fibril Associated Collagens with an Interrupted Triple Helix (FACITs)	These do not form fibrils themselves, but are found attached to the surface of pre-existing fibrils of the fibril forming collagens (Shaw and Olsen 1991).	**IX**	**vitreous, cartilage** and intervertebral disc
		XII	skin, **cartilage** and intervertebral disc
		XIV	skin, **cartilage** and intervertebral disc
		XV	basement membranes and **cartilage**
		XVI	skin, lung and arterial smooth muscle
		XVIII	most tissues, high levels in liver
		XIX	most tissues, basement membranes
		XX	corneal epithelium and tendon
		XXI	blood vessels and smooth muscle
		XXII	hair follicle
		XXVI	testis and ovary

Other Small Groups	Beaded filaments	**VI**	most tissues including **cartilage**
	Anchoring Fibrils for Basement Membranes	**VII**	anchor stroma to basement membranes in skin and cornea
	Transmembrane	**XXIII**	prostate carcinoma
		XIII	most tissues including skin
		XVII	skin and muscle

1.2.1.1 Major Articular Cartilage Collagens

- **Type II Collagen**

The major collagen type of articular cartilage is type II making up 90% of the collagen present (Duance *et al.,* 1999). This member of the fibril forming collagen family is made up of 3 identical polypeptide α1[II] chains. In mature cartilage type II collagen forms fibrillar networks with thicker fibrils in the deep layers of the cartilage and finer fibrils enriched in the surface layers (Aydelotte and Kuettner 1988). Type II collagen fibrils co-assemble with the minor collagen types XI and IX to form heterotypic fibrils (see Figure 1.2) (Vaughan *et al.,* 1988, and Mayne *et al.,* 1993). A model for packing of collagen molecules into fibrils is shown in Figure 1.3.

- **Type IX Collagen**

This member of the FACIT group of collagens is a heterotrimer with its 3 chains (α1[IX], α2[IX] and α3[IX]) being products of 3 different genes (Eyre and Wu 1995). The molecule has 3 triple helical collagenous domains (COL 1, 2 and 3), 4 non-collagenous domains (NC 1, 2, 3 and 4) (Duance *et al.,* 1999) and is stabilised by interchain disulphide bonds. Unusually for a collagen it has a chondroitin sulphate glycosaminoglycan chain attached to the NC 3 domain of the α2 chain (McCormick *et al.,* 1987) so is also classified as a proteoglycan. The proportion of this glycosaminoglycan bearing form varies between species and tissues. In the vitreous humour of the eye 100% is substituted (Bishop *et al.,* 1994), whereas in human articular cartilage only a small percentage is substituted (Diab *et al.,* 1996). Covalent cross links between the helical segments of type IX collagen and telopeptides of type II collagen suggest a bridging role for type IX between adjacent collagen fibres (see Figure 1.2) (Wu and Eyre 1989).

- **Type XI Collagen**

Type XI is a fibril forming collagen made up of 3 distinct α chains (α1[XI]α2[XI]α3[XI]). Together with type II and type IX collagen, type XI is co-assembled into heterotypic fibrils of articular cartilage (Mendler *et al.,* 1989). Type XI collagen contains 2 collagenous domains (COL 1 and 2) and 3 non-collagenous domains (NC 1, 2 and 3). The NC 2 domain forms a kink that produces an angle between COL 1 and 2. The association of type XI with type II and type IX is shown in Figure 1.2.

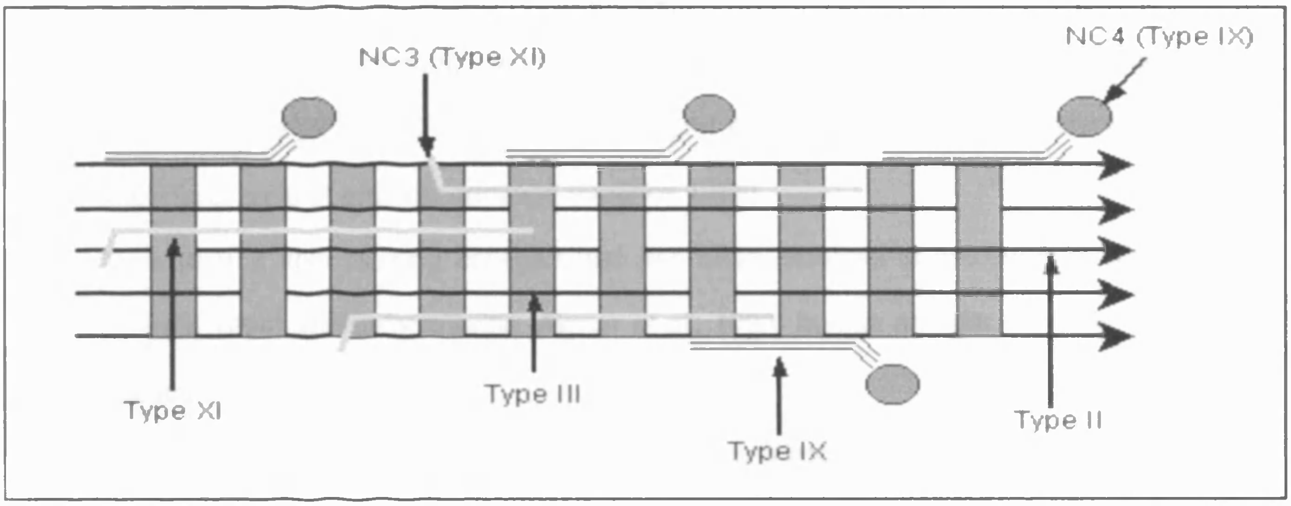

Figure 1.2 Schematic diagram showing potential interaction between collagen types II, IX and XI in articular cartilage (Eyre *et al.*, 1987)

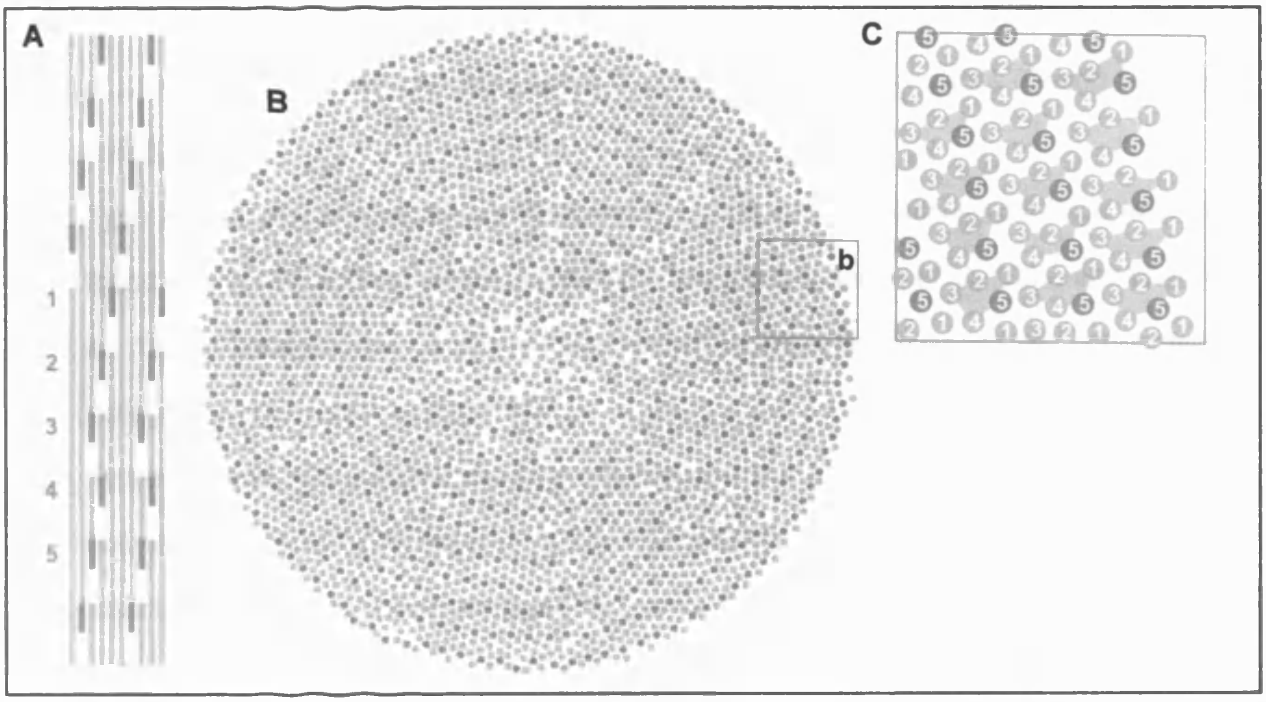

Figure 1.3 Molecular packing of collagen fibrils (A) Longitudinal view of collagen molecules in staggered array. Each molecule can be considered as consisting of 5 molecular segments 1 to 5, of which the short section 5 is shown in black. (B) Transverse section of the radial packing model (Hulmes *et al.*, 1995). Segments 5 (in black) are arranged in concentric layers separated by a distance of ~4nm. (C) Enlarged view of the boxed area in (b) showing molecules grouped together in the form of microfibrils. (Hulmes 2001)

1.2.1.2 Minor Articular Cartilage Collagens

The other collagens present in articular cartilage are types I, III, V, VI, X, XII, XIV and XV;

- Type I collagen is abundant in skin, bone and tendon but is only a very minor component of articular cartilage. It is a fibril forming collagen enriched in the 'lamina splendens' of the superficial zone of articular cartilage (Duance 1983).
- Type III is a fibril forming collagen that has been detected in both normal (Wotton and Duance 1994) and osteoarthritic (Aigner *et al.,* 1993) human articular cartilage, but only in the superficial zone.
- Type V collagen has a high homology to type XI and both are members of the fibril forming collagen family.
- Type VI collagen is found in most connective tissues including articular cartilage and forms microfibrils with a beaded appearance (Bruns 1984). It is involved in making up a pericellular fibrillar meshwork to protect the chondrocytes known as the chondron (Poole *et al.,* 1992). Type VI collagen expression is upregulated in osteoarthritis (Chang and Poole 1996).
- Type X collagen has a restricted distribution in normal human articular cartilage where it is found only in the calcified cartilage below the tide mark (Gannon *et al.,* 1991, and Walker *et al.,* 1995). Chondrocytes of osteoarthritic cartilage synthesise enhanced amounts of type X collagen (Von der Mark *et al.,* 1992)
- Types XII and XIV collagen are both members of the FACIT collagen family with partial homology to type IX (Dublet and Van der Rest 1991). Both types XII and XIV have been identified in bovine articular cartilage (Watt *et al.,* 1992) where they may carry a chondroitin sulphate chain and therefore exist in a proteoglycan form (Van der Rest and Dublet 1996).
- Type XV collagen has a widespread distribution in human tissues including heart, placenta and cartilage (Kivirikko *et al.,* 1995). The $\alpha 1$[XV] chain contains a highly interrupted collagenous region of 577 residues and noncollagenous amino- and carboxy-terminal domains of 530 and 256 residues, respectively (Kivirikko *et al.,* 1994).

In cartilage, the size and composition of the fibrils are dependent on the stage of development and the source of the tissue (Jan Bos *et al.,* 2001). In mammals, immature cartilage fibrils are of small (20nm) diameter (Keene *et al.,* 1995). In contrast, in adult tissue there are two populations of fibrils: small diameter fibrils that have surface associated type IX, and thick fibrils with very little type IX but the small proteoglycan decorin attached to their surface (see Figure 1.2) (Hagg *et al.,* 1998).

1.2.2 Cartilage Proteoglycans

Proteoglycans are extracellular matrix macromolecules present in varying amounts in all connective tissues and comprise over 20% of the dry weight of articular cartilage. They are composed of a protein core to which is attached one or more glycosaminoglycan (GAG) chains. Their properties and structure vary enormously allowing them to fulfil a diverse variety of biological roles.

Each proteoglycan contains one or two types of GAG chain as well as N- and O-glycosidically linked oligosaccharides, of the types found in glycoproteins. The core protein of proteoglycans is made on membrane bound ribosomes, and passes into the lumen of the endoplasmic reticulum (ER). The GAG chains are assembled on the protein core in the Golgi (Stryer 1995).

Proteoglycans can have limitless heterogeneity, because core proteins vary in MW (10,000-600,000kD), and GAG chains vary in length and number. Many proteoglycans, including aggrecan, syndecan and betaglycan contain two GAG chain types. The size and ratio of the GAG chains attached to proteoglycan core proteins may alter with aging and / or disease (Roughley and White 1980, and West *et al.,* 1999).

Proteoglycan core proteins can be sub-divided into various domains. All core proteins, by definition, contain a GAG substitution domain to which chondroitin, dermatan, keratan, heparan sulphates and heparin may be O-linked via serine or threonine residues, keratan sulphate may also be N-linked via asparagine residues. In addition to this, many proteoglycans are anchored to the cell surface, or to macromolecules in the extracellular matrix through specific domains in the core protein. Some core proteins contain domains with as yet unidentified roles and properties.

Many proteoglycans also interact with macromolecules of the extracellular matrix via their GAG chains. There are now more than 30 members of the proteoglycan superfamily, which can be grouped into families according to the GAGs they contain, where they are located, or the structure of their core proteins. In this discussion the proteoglycan family members covered in detail will only be those found in articular cartilage shown in Figure 1.5.

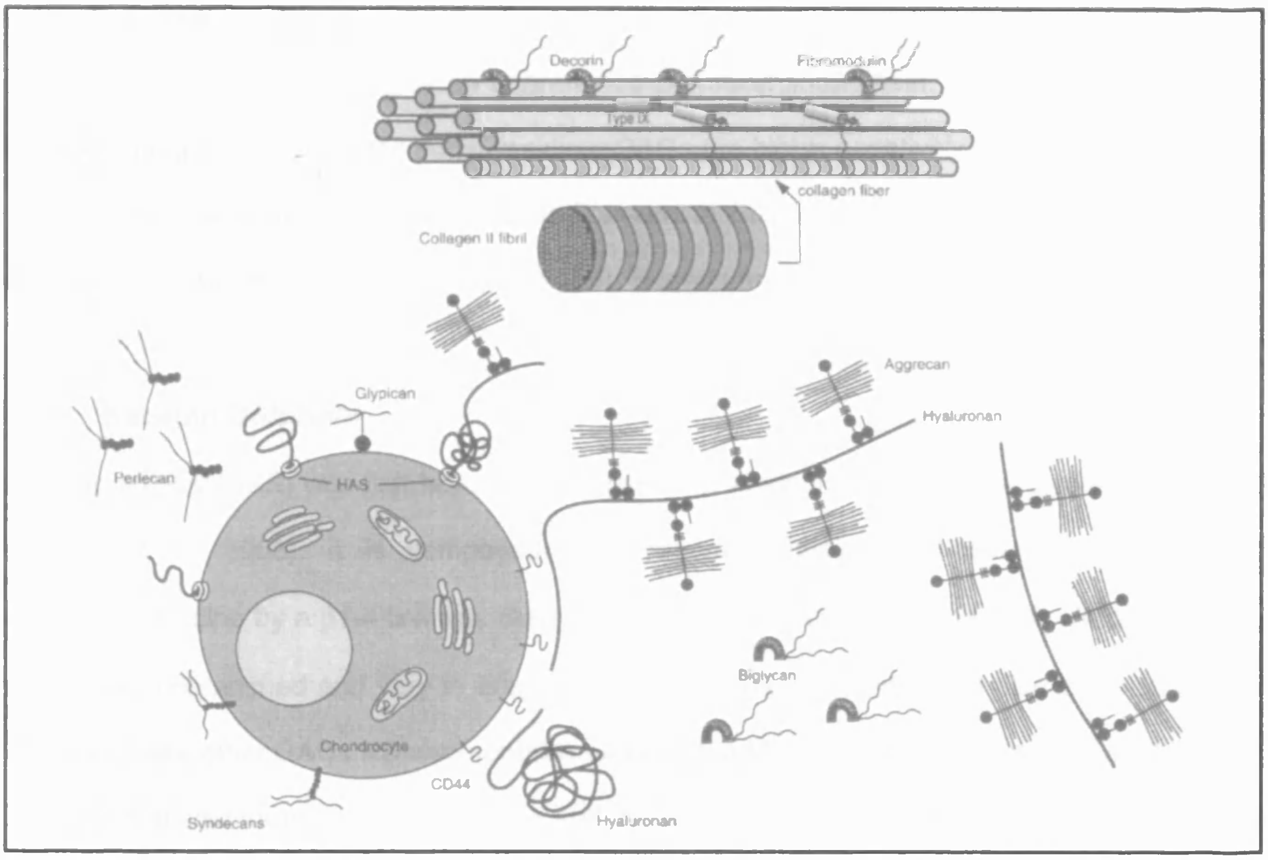

Figure 1.5 Overview of the proteoglycans present in cartilage. Depicted is a cartilage chondrocyte. Associated with the cell surface are the transmembrane spanning syndecan proteoglycans, the GPI-linked heparan sulphate proteoglycan, glypican, and two forms of hyaluronan, namely hyaluronan bound to the hyaluronan synthase (HAS) and hyaluronan tethered to CD44. Aggrecan binds to cell surface-associated hyaluronan within the further removed extracellular matrix. Several small proteoglycans namely decorin, fibromodulin and type IX collagen have been shown to form strong associations with cartilage collagen fibrils (collagen types II, IX and XI). Other proteoglycans such as biglycan and perlecan are also present within cartilage but their localisation and binding partners have not been firmly identified (Knudson and Knudson 2001).

1.2.2.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are long chains of linear polysaccharides formed from repeating disaccharide units. One of the sugars of the disaccharide is always a hexosamine, which is usually N-acetylated (N-acetylgalactosamine or N-acetylglucosamine), usually the amino sugar is sulphated. The second sugar unit is uronic acid either glucuronic or iduronic acid. The disaccharides polymerise to give an unbranched polysaccharide chain. As there are sulphate or carboxyl groups on most of the sugar residues GAGs are highly negatively charged. Several types of GAG chain are commonly recognised; Chondroitin Sulphate (CS), Dermatan Sulphate (DS), Keratan Sulphate (KS), Heparan Sulphate (HS), Heparin and Hyaluronan (HA).

- **Keratan Sulphate**

Keratan sulphate (KS) was first isolated from cornea in which it is the principal glycosaminoglycan (Meyer *et al.,* 1953). It is composed of a repeating chain of D-galactose joined to N-acetylglucosamine by a β1-4 linkage. Sulphation can occur on position 6 of each residue. The KS chains are unbranched and vary in length, from 5-10 units in intervertebral disc to 30-50 units in cornea. Unlike other GAGs KS also contains oligosaccharide branches (Dickenson *et al.,* 1990).

KS is also found in bone, cartilage and cornea (Hjertquist and Lemperg 1972, and Baker *et al.,* 1969). It is similar to the oligosaccharides of glycoproteins in the structure of its link region to proteoglycan core proteins and its sialic acid residues.

- **Chondroitin and Chondroitin Sulphates**

The name Chondroitin Sulphate (CS) derives from “Chondros” meaning cartilage from which it was first isolated (Krukenberg 1884), but they are also found in bone and heart valves (Mörner 1889, and Muir 1958) The GAG chain of chondroitin and chondroitin sulphate is composed of repeating units of D-glucuronic acid linked via β1-3 linkage to N-acetyl-D-galactosamine (Heinegård and Paulsson 1984). Chondroitin sulphates are sulphated varieties of chondroitin, where the ester sulphate group can be on carbon 4 giving chondroitin-4-sulphate (C-4-S) or on carbon 6 giving chondroitin-6-sulphate (C-6-S). Chondroitin sulphates may have either or both residues substituted and if neither are sulphated the GAG is chondroitin. Most chondroitin sulphate chains are copolymers of segments of one chondroitin sulphate, or chondroitin, interrupted by segments of another.

The highest chondroitin sulphate, or chondroitin, content is in cartilage and intervertebral disc (up to 10% wet weight) (Sztrolovics *et al.*, 2002). Nasal and epiphyseal cartilages contain a high proportion of C-4-S, whereas articular cartilage and the nucleus pulposus of the intervertebral disc, have a high C-6-S content. Aging articular cartilage contains a higher proportion of C-6-S than young cartilage (Roughley and White 1980, and West *et al.*, 1999).

❑ Dermatan Sulphate

The name Dermatan Sulphate (DS) derives from "Dermis" meaning skin, as this is its major location although it is also found in other connective tissues including heart valves, sclera, tendon, aorta, cornea and cartilage (Sztrolovics *et al.*, 2002). Dermatan sulphate is an isomer of chondroitin-4-sulphate in which the D-glucuronic acid undergoes epimerisation to form L-iduronic acid. Therefore the GAG chain of dermatan sulphate is composed of repeating units of L-iduronic acid linked via an α1-3 linkage to N-acetyl-D-galactosamine. L-Iduronic acid residues may be sulphated in the 2 position. Although dermatan sulphate is often found in GAG chains interspersed with chondroitin sulphate units (Rodén 1980), only one iduronic acid residue is required for the chain to be dermatan sulphate.

❑ Heparin and Heparan Sulphate

The names Heparin and Heparan Sulphate (HS) derive from "Hepas" meaning liver, from which they were first isolated (Oldberg *et al.*, 1977). The chains of both GAGs are composed of repeating units of either D-glucuronic or L-iduronic acid linked via a β1-4 linkage to N-acetyl-D-glucosamine, both residues can be sulphated at the O- and N- positions. The GAG chain is a copolymer of the two types of disaccharide and is the most complex of all the GAGs. Heparan sulphate and heparin differ in their N-Sulphate and N-Acetyl contents and their localisation. Heparan sulphate contains a higher acetylated glucosamine than heparin and is found in basement membranes and components of cell surfaces (Stevens and Austen 1989). In contrast, heparin contains a higher proportion of N-sulphate (up to 90%). It also plays a role in preventing the coagulation of blood, as it is abundant in mast cell granules (Le Trong *et al.,* 1987).

- **Hyaluronan**

Hyaluronan (HA) is the simplest GAG its name derives from "hyaloid" meaning vitreous as it is found in the vitreous humour of the eye, as well as the extracellular matrix of connective tissue and synovial fluid (Meyer and Palmer 1934). The hyaluronan chain is composed of repeating units of D-glucuronic acid linked via β1-3 linkage to N-acetyl-D-glucosamine. It is a regular repeating sequence of up to 25,000 non-sulphated disaccharide units with a MW of 100,000 - 10,000,000.

Hyaluronan is found in variable amounts in the connective tissues of adults but is more prevalent in embryos where it facilitates cell migration during tissue morphogenesis (Toole *et al.,* 1977). In some connective tissues, such as umbilical cord and vitreous body it is the main GAG others, like cartilage, have a relatively low hyaluronan content (1%). Unlike all other GAGs it contains no sulphate and is not found covalently attached to proteins to form proteoglycans. However, it does form non-covalent complexes with proteoglycans in the extracellular matrix.

Hyaluronan has been used as the basis for a number of biodegradable polymers including Hyaff® 11, which is currently under investigation as a biomaterial onto which chondrocytes can be seeded and cultured for insertion into cartilage tears (Grigolo *et al.,* 2001a, and Grigolo *et al.,* 2001b).

Some GAGs have been shown to bind growth factors affecting their potency in some cases growth factor activity is critically dependent upon GAGs as co-receptors. For example heparan and dermatan sulphate bind hepatocyte growth factor / scatter factor and effect a conformational change in the molecule altering its activity (Lyon *et al.,* 2002).

1.2.2.2 N- and O- Linked Oligosaccharides

Proteoglycan molecules often contain oligosaccharides linked through O- and N- glycosidic bonds. Since O-glycosidic oligosaccharides are structurally similar to the keratan sulphate type II linkage region it has been suggested that they are derived from the same carbohydrate core by differential processing (Lohmander *et al.*, 1980, De Luca *et al.*, 1980, and Nilsson *et al.*, 1982). The role of oligosaccharides in the functions of proteoglycans is fairly unknown, but there is evidence that at least in the large proteoglycans they may cover the protein core maintaining the structure of the proteoglycans and protecting them against proteolytic activities (Bernard *et al.*, 1983)

1.2.2.3 Linkage and Synthesis of GAG Chains and Oligosaccharides on the Proteoglycan Core Protein

With the exception of hyaluronan, which appears to be assembled as a free polysaccharide chain (Prehm 1983), all GAGs are synthesised as proteoglycans i.e. covalently linked to a core protein.

- The linker sequence for chondroitin (sulphates), dermatan sulphate, heparan sulphate and heparin forms a bridge between the GAG chain proper and the polypeptide core as depicted in Figure 1.4 (Rodén 1980). The linker sequence in the GAG chain is coupled via an O-glycosidic bond to a serine or threonine in the core protein via the hydroxyl group of the amino acid (Figure 1.4) (Muir and Hardingham 1975).
- Linkage of keratan sulphate and oligosaccharides to the proteoglycan core protein can occur via N- or O- linked glycosylation. These two forms of linkage are used to distinguish the two forms of keratan sulphate found in mammals and divides oligosaccharides into N- and O-linked varieties. Corneal keratan sulphate (Type I Keratan Sulphate) and N-linked oligosaccharides are linked to an asparagine in the core protein via mannose and N-acetyl glucosamine residues (Bray *et al.*, 1967, Choi and Meyer 1975, and Nilsson *et al.*, 1983). Skeletal keratan sulphate (Type II Keratan Sulphate) and O-linked oligosaccharides are linked to serine or threonine residues via an N-acetyl galactosamine residue (Hoffman and Mashburn 1967, Choi and Meyer 1975, Stuhlsatz *et al.*, 1989, and Hascall and Midura 1989).

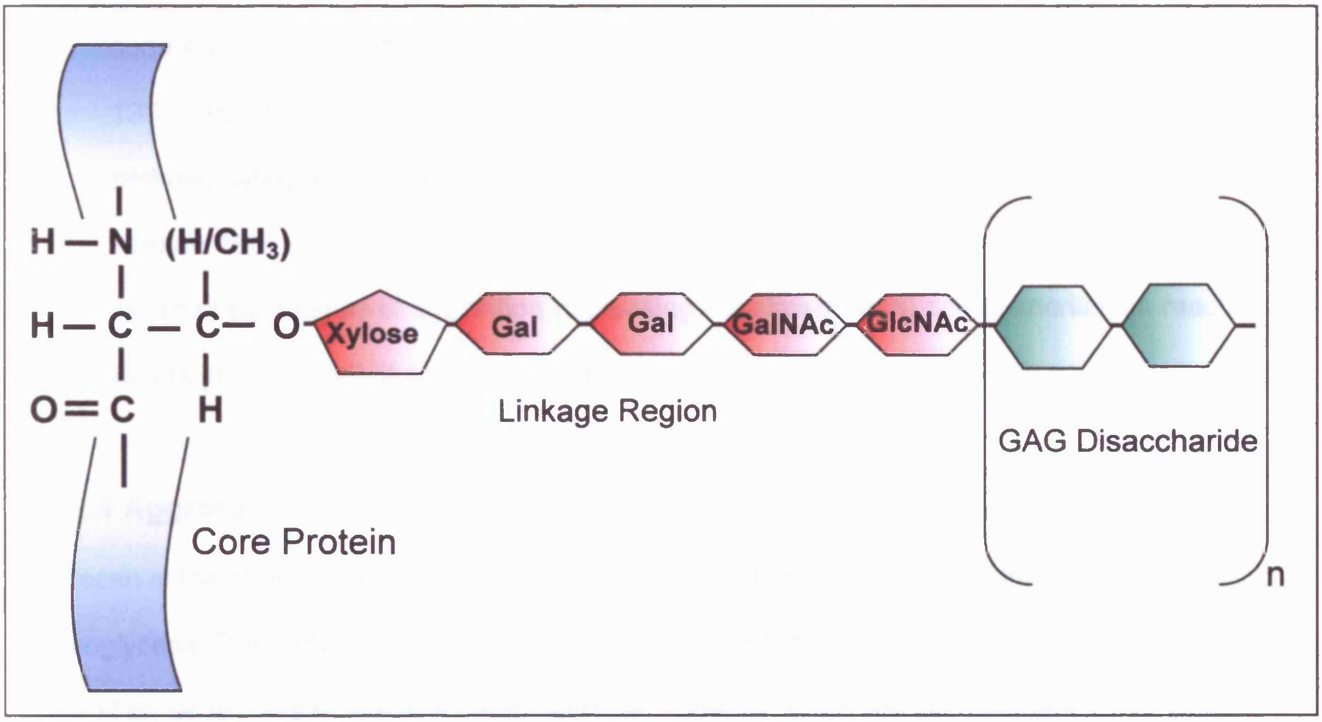

Figure 1.4 Diagram to show the linkage of chondroitin, dermatan and heparan sulphate as well as heparin to the core protein of proteoglycans through Xylose - Galactose - Galactose - N-acetyl Galactosamine – N-acetyl Glucuronic Acid which couple via an O-glycosidic bond to a serine or threonine in the core protein

- Hyaluronan is unique among GAGs since it appears to be synthesised as a free polysaccharide chain at the plasma membrane (Prehm 1984). The carbohydrate being built inside the membrane and pushed out into the extracellular space, the non-reducing end leading (Prehm 1983). Three different hyaluronan synthases have been cloned (Spicer *et al.,* 1997). All GAGs other than hyaluronan are synthesised attached to the core protein of a proteoglycan, in the Golgi, by addition of monosaccharide units from the appropriate UDP-sugars to the non-reducing ends of nascent polysaccharide chains (Lindahl 1976, Rodén 1970, and Silbert and Reppucci 1976). Chain elongation is initiated by xylosylation of serines, catalysed by a xylosyl transferase using UDP-xylose as a sugar donor (Kjellin and Lindahl 1991). Whilst still in the Golgi many polymerised sugar residues are covalently modified by a series of sulphation (increasing negative charge) and epimerisation reactions (alters arrangement of atoms on sugar ring).

1.2.2.4 Aggrecan

Aggrecan is the major proteoglycan of articular cartilage and is a member of the hyalectin family of proteoglycans. Other Hyalectin or Lectican family members include versican (secreted by fibroblast cells) (Tan *et al.,* 1993), brevican and neurocan (both of which are predominantly expressed in adult brain) (Kurazono *et al.,* 2001, and Gary *et al.,* 2000). All members of the hyalectin family have the ability to bind hyaluronan at their amino-terminal end and other matrix glycoproteins through their carboxy terminal G3 C-type lectin domains (Halberg *et al.,* 1988, Watanabe *et al.,* 1998, and Zhang *et al.,* 1998).

The core protein of aggrecan has a molecular weight of around 250-300kD and consists of three globular domains, G1, G2 and G3, interspersed by rod-like segments. Between G1 and G2 is the interglobular domain (IGD). Keratan sulphate and chondroitin sulphate glycosaminoglycan attachment domains are located between the G2 and G3 domains (see Figure 1.6) (Paulsson *et al.,* 1987). The amino-terminus comprises the G1 globular domain, which non-covalently interacts with hyaluronan and link protein (Kohda *et al.,* 1996). The G1 domain contains three looped subdomains, A, B and B'. Both the B and B' loops form disulphide bonding double loop structures called proteoglycan tandem repeat (PTR) units (Kohda *et al.,* 1996). The PTR loop contains the functional site of the binding of aggrecan to hyaluronan (Iozzo 1998). Aggrecan binds to link protein in a 1:1 ratio (Hardingham and Muir 1973, and Mörgelin *et al.,* 1994). The G1-hyaluronan-link protein complex is very stable essentially immobilising the aggrecan within the cartilage matrix. The secondary structure is significant and the hyaluronan binding region (comprising the G1 domain) no longer binds hyaluronan under reducing conditions. Oegema suggested that newly synthesised aggrecan is not immediately incorporated into aggregates, and furthermore that this delayed aggregation is age dependent (Oegema 1980). Moreover Bayliss *et al.,* suggested that newly synthesised aggrecan is processed in pools with different capacities for aggregation with hyaluronan and stabilisation by link protein, and that tissue compartments, possibly defined by extracellular pH, show differences with age and with disease state (Bayliss *et al.,* 2000).

The G2 globular domain is separated from the G1 by a linear interglobular domain (IGD), which has two homologous PTRs but does not itself bind to hyaluronan. The large GAG substituted domain is located between globular domains G2 and G3 and contains both chondroitin sulphate and keratan sulphate binding regions. Some chains are attached outside of this region but only a small percentage of the total number of GAGs present.

The average aggrecan monomer has a molecular weight of 2.5 million Dalton, with up to 90% of its mass being contributed by approximately 100 chondroitin sulphate chains, 30 keratan sulphate chains and a number of N- and O- linked oligosaccharides that are covalently attached to the core protein (Nilsson *et al.,* 1982, De Luca *et al.,* 1980, Lohmander *et al.,* 1980, and Hardingham 1986).

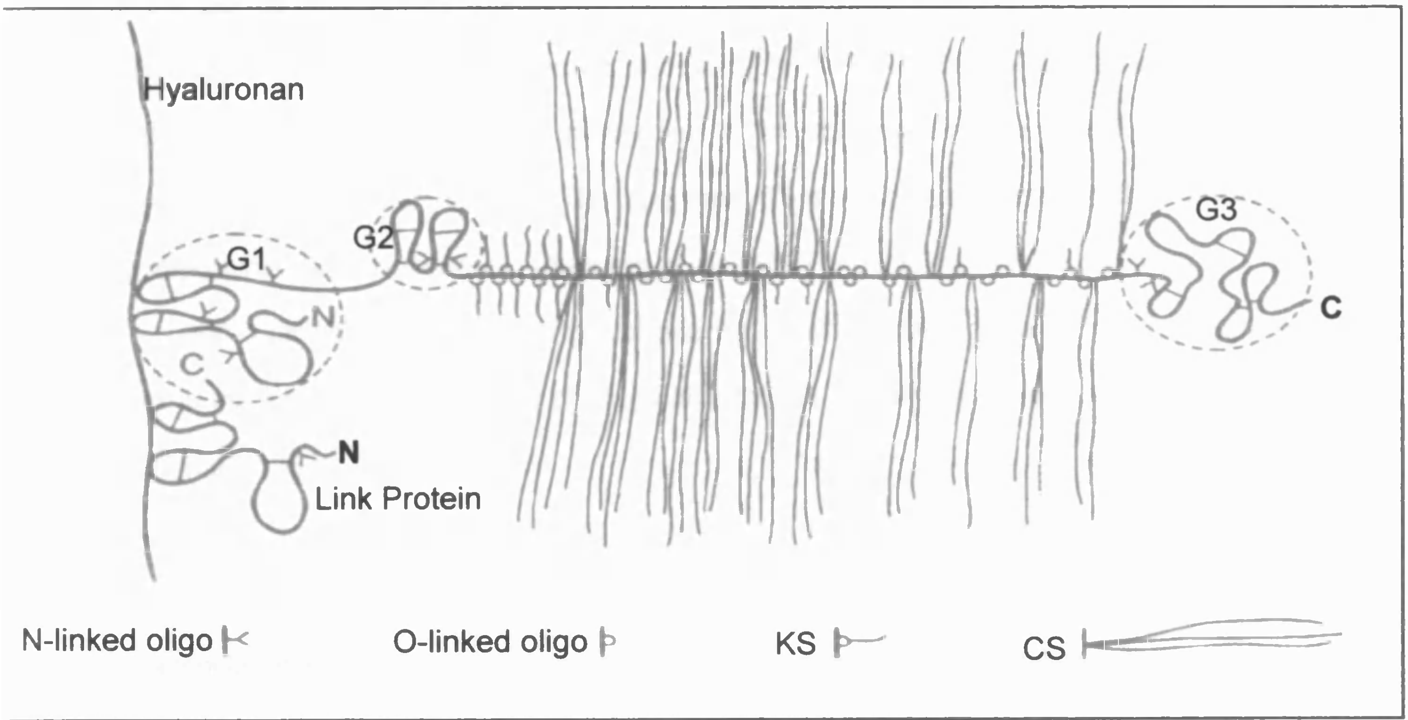

Figure 1.6 Schematic representation of aggrecan, its domain structure and non-covalent interaction with hyaluronan stabilised by link protein (Vertel 1995)

The carboxy terminus comprises the G3 domain that includes an Epidermal Growth Factor (EGF)-like module, a C-type lectin module and a complement regulatory protein module. Alternative splicing of the G3 domain has been reported (Murdoch *et al.*, 2002) and the spliced form of the G3 domain lacking the EGF motifs is common in chondrocytes maintained in cell culture for even short periods of time (Grover and Roughley 1993). Roles have been suggested for the G3 domain in modulation of GAG attachment to the core protein and in regulation of core protein secretion (Chen *et al.*, 2002a). The aggrecan G3 domain may interact with extracellular matrix components including tenascin (Aspberg *et al.*, 1997) and fibulins (Aspberg *et al.*, 1999, and Olin *et al.*, 2001) as well as cell surface glycolipids (Miura *et al.*, 1999). Around half the aggrecan molecules in the extracellular matrix of cartilage lack the G3 domain owing to proteolytic cleavage (Paulsson *et al.*, 1987).

Aggrecan is packed into the cartilage matrix at up to 10 - 20% of its free solution volume (Vertel 1995). The GAG chains on each aggrecan monomer have a high concentration of negative charges associated with their carboxyl groups and extensive sulphate substitutions. These are held within the cartilage extracellular matrix by non-covalent association of aggrecan monomers with hyaluronan through the G1 domain and stabilised by link protein to form supramolecular aggregates (Mörgelin *et al.*, 1994). These form a highly compressible and resilient tissue when fully hydrated.

One of the first pathological features of joint degeneration is loss of aggrecan from the cartilage matrix preceding both overt collagen catabolism and joint erosion by some time. Following joint injury and subsequent joint disease there is a loss of aggrecan metabolites from the cartilage matrix into the synovial fluid (Lohmander 1991). Cleavage sites for many families of proteases are found along the length of the aggrecan core protein as shown in Table 1.2 (Sandy *et al.*, 1992). Degradation of aggrecan is further discussed in Section 1.5.

Table 1.2 A selection of the cleavage sites within the aggrecan core protein, the enzymes which utilise them and the resultant neoepitopes.

ENZYME	CLEAVAGE SITE(S)		REFERENCES
MMPs including 1, 2, 3, 7, 9, 10, 13, 19 and 20, and Cathepsin B	IPEN^{341} – ^{342}FFGV	CLEAVAGE SITES WITHIN THE INTERGLOBULAR DOMAIN OF THE AGGRECAN CORE PROTEIN	Fosang *et al.*, 1991, 1992, 1993, 1994, 1996 and 1998 Lark *et al.*, 1995 Stracke *et al.*, 2000 Little *et al.*, 1999 Mort *et al.*, 1998
Cathepsin B	NFFG^{344} – ^{345}VGGE		Fosang *et al.*, 1992 Mort and Buttle 1997
MMP-8 and 14	TEGE^{373} – ^{374}ARGS IPEN^{341} – ^{342}FFGV		Fosang *et al.*, 1994 Fosang *et al.*, 1993 Fosang *et al.*, 1998
ADAMTS-1, -4 and -5	TEGE^{373} – ^{374}ARGS		Kuno *et al.*, 2000 Sandy *et al.*, 1991a Tortorella *et al.*, 1999 Tortorella *et al.*, 2000a and b Tortorella *et al.*, 2002 Abbaszade *et al.*, 1999 Rodriguez-Manzaneque *et al.*, 2002 Sandy *et al.*, 2002
MMP-1, -7, -8 & –13	TSED^{441} – ^{442}LVVQ	CLEAVAGE SITES WITHIN THE CHONDROITIN AND KERATAN SULPHATE ATTACHMENT REGIONS OF THE AGGRECAN CORE PROTEIN	Fosang *et al.*, 1996 Fosang *et al.*, 1994 Fosang *et al.*, 1991
MMP-1	CRFG^{656} – ^{657}ISAV		Fosang *et al.*, 1993
Cathepsin D	VEEW^{680} – ^{681}IVTQ		Handley *et al.*, 2001
ADAMTS-4 and -5	ELE^{1545} – ^{1546}GRG KEEE^{1714} – ^{1715}GLGS TAQE^{1819} – ^{1820}AGEGE ISQE^{1919} – ^{1920}LCQR		Tortorella *et al.*, 2000a and b Tortorella *et al.*, 2002 Lee *et al.*, 2002

1.2.2.5 Small leucine rich proteoglycans (SLRPs)

All members of the small leucine rich proteoglycan family except chondroadherin and PRELP are able to bind collagen and are found along the surfaces of collagen fibrils in cartilage. There are three classes of small leucine rich proteoglycans and members from each class are found in cartilage.

- **Class I**

Class I includes decorin and biglycan both of which are found in articular cartilage and bind TGFß sequestering its mitogenic activity (Yamaguchi *et al.,* 1990, and Schönherr *et al.,* 1998). In articular cartilage biglycan is found in the pericellular matrix while decorin is found in the interterritorial matrix (Bianco *et al.,* 1990). The core proteins of biglycan and decorin are 50-60% homologous in their protein sequence, both have MW of around 37,000 with an extra 30,000 being added by the GAG chains (Hocking *et al.,* 1998). Each of the core proteins is divided into 4 domains. At their amino-termini are GAG attachment regions, decorin carries a single chondroitin or dermatan sulphate chain whereas biglycan carries two such chains (Cheng *et al.,* 1994). A metallothioneine-like domain containing a disulphide bond between 2 cysteine residues divides the GAG attachment region from the leucine rich repeat domain. A 49 amino acid long region unique to these two proteoglycans forms the carboxy-terminus of both decorin and biglycan (Kresse *et al.,* 1993).

Decorin is associated with collagen fibrils as a decorating proteoglycan (Gallagher *et al.,* 1983, Scott 1988, and Vogel *et al.,* 1984). It is described as a horseshoe shaped molecule, and this arched structure as well as the dimensions of the curve, supports a model for decorin interaction with a single triple helix of collagen (Vynios *et al.,* 2001). Since the concave surface of the decorin core protein is presumed to bind in the gap zone of the collagen fibril, the GAG chain located near the amino-terminus of decorin would be free to maintain fibril-fibril spacing. The message level for decorin in cartilage is by far the most abundant of all SLRP family members and shows increases with increasing age in human articular cartilage (Melching and Roughley 1989).

The tissue localisation and the potential interaction with other cartilage matrix components have been less clearly defined for biglycan although is has been suggested as a positive regulator of bone formation and bone mass (Xu *et al.,* 1998).

□ **Class II**

Class II SLRPs include fibromodulin, lumican, osteoadherin, keratocan and proline arginine-rich and leucine-rich repeat protein (PRELP), of which all except keratocan and osteoadherin are expressed in cartilage (Grover *et al.,* 1995, Sommarin *et al.,* 1998, and Melching and Roughley 1989). This group can be further sub-divided into three distinct subfamilies based on protein sequence homology. Fibromodulin and lumican constitute the first subfamily and exhibit ~48% protein sequence identity (Hocking *et al.,* 1998); keratocan and PRELP constitute the second subfamily with ~55% protein identity (Bengtsson *et al.,* 1995), whereas osteoadherin constitutes a distinct subfamily with 37-42% protein identity to other class II members (Somarin *et al.,* 1998).

All class II SLRPs share an identical cysteine rich region followed by a leucine rich repeat region. Class II members are primarily substituted with keratan sulphate chains, however polylactosamine (an unsulphated keratan sulphate) is found on both fibromodulin and keratocan (Plaas *et al.,* 1993, and Corpuz *et al.,* 1996).

PRELP exhibits protein sequence similarity to both lumican and fibromodulin and has four potential N-linked glycosylation sites. Thus although it is a member of the SLR proteins, it apparently functions as a cartilage matrix protein with the capacity for matrix organisation (Bengtsson *et al.,* 1995).

Fibromodulin carries up to four keratan sulphate chains (Plaas *et al.,* 1990) and has the ability to decorate the surface of collagen fibers and therefore may regulate fibril diameter (Hedlund *et al.,* 1993). The message levels for fibromodulin and lumican show increases with increasing age in human articular cartilage (Melching and Roughley 1989).

Lumican is the major keratan sulphate proteoglycan in the cornea, but also shows widespread distribution in connective tissues, including articular cartilage (Melching and Roughley 1989, and Ying *et al.,* 1997). It is interesting that in young cartilage lumican is found as a keratan sulphate proteoglycan while after IL-1 treatment chondrocytes synthesise and secrete the lumican protein devoid of GAG substitutions (Melching and Roughley 1999).

- **Class III**

Epiphycan (PG-Lb) and mimecan (osteoglycan) which exhibit only ~40% protein sequence identity are the two members of the class III SLRPs expressed in articular cartilage (Shinomura and Kimata 1992). These proteoglycans can be distinguished from other SLRPs by a unique cysteine-rich region consensus sequence (CX_2CXCX_6C) and by the presence of only six leucine-rich repeats (Johnson *et al.,* 1997).

Epiphycan derives its name from epiphyseal cartilage where it was first isolated (Johnson *et al.,* 1997). The GAG attachment region is composed of two serine residues with a consensus structure similar to aggrecan, decorin and biglycan. This region may contain either chondroitin sulphate or dermatan sulphate. Epiphycan can also be secreted as a glycoprotein. The expression of epiphycan during development of the growth plate lags behind that of aggrecan, and is excluded from both the layer of presumptive articular cartilage and the hypertrophic zone (Johnson *et al.,* 1999).

Mimecan is abundantly expressed in sclera and cornea, but can also be found in non-ocular tissues as a non-sulphated glycoprotein (Funderburgh *et al.,* 1997, and Madisen *et al.,* 1990). Mimecan may play a role in controlling cell growth as illustrated by the ability of growth factors and cytokines to modulate its expression (Shanahan *et al.,* 1997). At present there is no published information on the mechanisms by which mimecan exerts its biological function (Tasheva 2002).

1.2.2.6 Perlecan

Perlecan derives its name from its rotary shadowing appearance; a string of pearls (Paulsson *et al.,* 1987, and Yurchenco *et al.,* 1987). It is a large heparan sulphate proteoglycan with a core protein of ~467kD that is found in all basement membranes and a variety of other specialised tissues including the synovium, cartilage and developing bone (Sundaraj *et al.,* 1995, and Handler *et al.,* 1997).

Perlecan is a complex molecule made up of five distinct domains with only domain I being unique (Iozzo *et al.,* 1994). Domain II shares homology with low-density lipoprotein receptor. Domain III is similar to the amino-termini of the short arms of laminins A and B. Domain IV is homologous to the Neural Cell Adhesion Molecule (N-CAM) (Tapanadechopone *et al.,* 1999). At the carboxy terminus of perlecan domain V contains two epidermal growth factor (EGF)-like repeats and two Leucine Arginine Glutamine (LRE) tripeptides.

Perlecan can undergo self-aggregation and can also interact with laminin, nidogen and fibronectin (Hopf *et al.,* 1999). Integrins have been proposed to function as cell surface receptors for perlecan. In adult articular cartilage perlecan is enriched in the pericellular matrix (Sundarraj *et al.,* 1995).

Knockout mice lacking the Hspg2 gene (encoding perlecan) showed disorganisation in their cartilage chondrocyte arrangement as well as defective endochondral ossification. The cartilage matrix itself was deficient in collagen fibrils and glycosaminoglycans, and what was present was severely disorganised suggesting a role for perlecan in matrix structural organisation (Hirasawa *et al.,* 1999). An alternatively spliced form of perlecan has recently been identified but its' role has yet to be elucidated (Dodge *et al.,* 2001).

1.2.2.7 Proteoglycan-4 (PRG-4)

A novel proteoglycan synthesised by superficial zone chondrocytes of articular cartilage was first identified and named superficial zone protein by Schumacher *et al.,* in 1994. This protein is now known as PRG-4 and is secreted into experimental medium or synovial fluid *in vivo,* with little incorporation into the extracellular matrix. It has a molecular weight of 345kD and a multidomain structure comprising structural motifs at its amino-and carboxy-terminals including vitronectin-like domains, somatomedin-B type domains, an aggregation domain and a heparin-binding domain, as well as large and small mucin-like domains substituted with O-linked oligosaccharides (Flannery *et al.,* 1999a). Due to its location and deduced structure PRG-4 has been suggested to play roles in cell proliferation, cytoprotection, lubrication, self-aggregation and matrix binding (Flannery *et al.,* 1999a).

1.2.2.8 Cell Surface Proteoglycans

Chondrocytes express cell surface proteoglycans, members of the transmembrane family of syndecans and the phosphodidylinositol linked heparan sulphate proteoglycans glypicans (Grover and Roughley 1995, and Hall and Miyake 2000).

- **Syndecans**

  The syndecan family contains four members; syndecan-1 (syndecan), syndecan-2 (fibroglycan), syndecan-3 (N-syndecan) and syndecan-4 (ryudocan or amphyglycan), all of which are transmembrane heparan sulphate proteoglycans (Bernfield *et al.,* 1992). Syndecan family members are type-I integral membrane proteins with homologous transmembrane and cytoplasmic domains (Pacifici and Molinaro 1980, and Saunders *et al.,* 1989). Syndecans may carry two or more heparan sulphate chains, alone or in combination with chondroitin sulphate, and these give them the potential to interact with basic Fibroblast Growth Factor (FGF), and modulate its interaction with its signalling receptor (Lyon *et al.,* 2002). All syndecans exhibit cell-type specific distribution, e.g. analyses of mRNA from articular chondrocytes demonstrated that message for syndecan-4 was of the highest abundance with some low level expression of syndecan-2 also detectable (Grover and Roughley 1995). In contrast syndecan-3 is expressed briefly and specifically during early stages of chondrogenesis (Hall and Miyake 2000). During maturation of the growth plate expression of syndecan-3 persists in the zone of proliferating chondrocytes, but is not detected in the layer of presumptive articular chondrocytes (Shimazu *et al.,* 1996).

- **Glypicans**

  Glypicans are a family of cell surface transmembrane heparan sulphate proteoglycans comprising 5 members: glypican-1 (glypican), glypican-2 (cerebroglycan), glypican-3 (OCI-5), glypican-4 (K-glypican) and glypican-5. All members of this family possess an extracellular region with a GAG attachment site as well as a carboxy-terminal GPI-anchor. Glypican family members are selectively expressed in different cell types and are mainly targeted to apical surfaces of cells (Tumova *et al.,* 2000). Glypican-1 is expressed in cartilage (Grover and Roughley 1995)

1.2.3 Other Extracellular Matrix Molecules

In recent years numerous cartilage proteins that are neither collagens nor proteoglycans have been identified and characterised.

1.2.3.1 Fibronectin

Fibronectin consists of two 250kD polypeptide chains that are linked by a disulphide bond near to the carboxy-terminus. It is a rod like molecule with several domains that can specifically interact outside the cell. The various globular domains play different roles in extracellular matrix interactions with different domains binding to collagens, heparin, and heparan sulphate or cell surface receptors (see Potts and Campbell 1996 for review). The specific cell-binding domain of fibronectin contains an Arginine Glycine Aspartate (RGD) amino acid sequence which is involved in cell attachment through integrins (Main *et al.,* 1992). Alternative splicing of fibronectin gene transcripts results in different protein isoforms. In adult canine and equine articular cartilage 50-80% of the fibronectin transcripts have a unique splicing pattern (Macleod *et al.,* 1996). Certain parts of the fibronectin molecule are susceptible to proteolysis resulting in a series of fragments. Fragments of fibronectin may regulate cartilage metabolism possibly through increasing levels of catabolic cytokines, which in turn upregulate matrix protease expression and enhance degradation (Homandberg 1999). The carboxy-terminal domain of fibronectin has recently been shown to bind to ADAMTS-4 (see Sections 1.3.5 and 1.5.2) inhibiting its ability cleave aggrecan, however the physiological relevance of this is not known (Hashimoto *et al.,* 2004).

1.2.3.2 Tenascins

Five members of the tenascin family have been identified tenascin-C (Erikson and Iglesias 1984), tenascin-R, tenascin-W, tenascin-X and tenascin-Y (Jones and Jones 2000). Tenascin-C is said to have a highly symmetrical structure taking the form of a hexabrachion i.e. six arms emanating from a central core. This structure is formed from six polypeptide chains linked at their amino-termini via a domain known as the Tenascin Assembly (TA) domain. The overall structure of all the tenascin proteins is the same, although tenascin-C is the only one of the family known to form hexabrachions (Jones and Jones 2000). Tenascin-C has been found in cartilage, skin and bone marrow (Mackie 1997). Tenascin-R is expressed exclusively in the central nervous system (Rathjen *et al.,* 1991). Tenascin-W is expressed predominantly in nervous tissue (Weber *et al.,* 1998). Both Tenascin-X and -Y are expressed in connective tissues (Matsumoto *et al.,* 1994, and Tucker *et al.,* 1999). In connective tissues tenascins interact with and can be cleaved by matrix metalloproteinases (Streuli 1999). Tenascin-C and tenascin-R both bind with high affinity to the hyalectin family of proteoglycans with brevican-tenascin complexes in the brain and aggrecan-tenascin complexes in cartilage (Aspberg *et al.,* 1995, and Aspberg *et al.,* 1997).

1.2.3.3 Cartilage Intermediate Layer Protein (CILP)

Cartilage Intermediate Layer Protein (CILP) has a calculated molecular mass of 78.5kD. It contains 30 cysteine residues and six putative N-glycosylation sites. Ten percent of its total mass is composed of N-linked oligosaccharides (Lorenzo *et al.,* 1998b). The tissue distribution of CILP is limited to cartilage and specifically the intermediate zone mainly in interterritorial areas (Lorenzo *et al.,* 1998b). Levels of the protein vary between cartilage types with tracheal cartilage having low levels and rib cartilage particularly high levels. CILP forms part of a group of matrix components whose expression is enhanced in the early stages of osteoarthritis and in aging cartilage (Lorenzo *et al.,* 1998b). The reasons behind CILP's specific distribution pattern and its exact role have yet to be elucidated. However an autoimmune response to CILP has been suggested to be involved in the pathogenesis of inflammatory joint destruction (Kato *et al.,* 2001).

1.2.3.4 Cartilage Oligomeric Matrix Protein (COMP)

COMP was initially identified in cartilage and its major site of expression is around chondrocytes, but it has also been purified from a number of other tissues including tendon, ligament and meniscus (Hauser *et al.,* 1995). It is a 524kD homopentameric extracellular glycoprotein (Hedbom *et al.,* 1992) that belongs to the thrombospondin family of proteins (Oldberg *et al.,* 1992). Each COMP monomer is composed of an amino-terminal cysteine-rich domain, four EGF-like domains, eight calmodulin-like repeats and a carboxy-terminal globular domain (Delot *et al.,* 1998). The cysteine rich domain is responsible for the assembly of monomers into pentamers via interchain disulphide bonds. The carboxy-terminal globular domain may be involved in binding cells (e.g. chondrocytes) and proteins in the extracellular matrix (Chen *et al.,* 2002b). Levels of COMP in serum or synovial fluid samples have been used as a marker of cartilage degeneration in both rheumatoid and osteoarthritis patients (Saxne and Heinegard 1992, Vilím *et al.,* 1997, and Vilím *et al.,* 2002), however its exact biological role has yet to be ascertained, although it has been suggested to play a role in modulation of chondrocyte phenotype (Chen *et al.,* 2002b).

1.2.3.5 Matrilins

Matrilin-1 was the first member of this now four strong family of cartilage proteoglycan associated proteins to be identified (Paulsson and Heinegård 1981). Matrilins all share a common domain structure composed of von Willebrand factor A domains (vWFA), EGF-like domains and a coiled coil α-helical module (Deak *et al.,* 1999). The best-characterised member of the family is matrilin-1, which was initially identified due to its tight association with aggrecan (Paulsson and Heinegård 1981), and has since been shown to covalently bind to the chondroitin sulphate region of the aggrecan core protein (Hauser *et al.,* 1996). Matrilin-1 has also been shown to associate with cartilage collagen fibrils (Winterbottom *et al.,* 1992). Based on its association with aggrecan and collagen fibrils it has been speculated that matrilin-1 functions in connecting the various supramolecular assemblies in cartilage (Deak *et al.,* 1999) and the other members of the matrilin family are thought to have similar roles. Matrilin-1 gene expression, not normally seen, has been detected in articular cartilage chondrocytes from patients with osteoarthritis (Okimura *et al.,* 1997) and increased serum levels of matrilin-1 can be used as a diagnostic marker for osteoarthritis (Meulenbelt *et al.,* 1997, and Okimura *et al.,* 1997). Matrilin-1 concentrations in serum are also elevated in relapsing polychondritis as well as in active rheumatoid arthritis (Saxne and Heinegård 1989 and 1995).

1.3 Cartilage Matrix Proteases and their Inhibitors

In the course of normal matrix turnover, and in disease states such as arthritis, articular cartilage matrix macromolecules can be cleaved and eventually broken down by members of several enzyme families including; serine proteases, cathepsins, MMPs, ADAMs and ADAMTSs.

1.3.1 Serine Proteases

Serine proteases are a large group of related proteins which function to hydrolyse other proteins. All serine proteases have the same mechanism of action. Their activity is dependent on a serine residue at the active site, hence their name. In mammals serine proteases play roles in many important processes including digestion, blood clotting and the complement system (Voet *et al.,* 2002). The active site of serine proteases is a cleft where the polypeptide substrate binds. The enzymes are inhibited by serpins, which inhibit the activity of their respective serine protease by mimicking the three-dimensional structure of the normal substrate of the protease (Imamura *et al.,* 2005). Serine proteases including plasmin, tissue plasminogen activator and urokinase-type plasminogen activator, plasma kallikrein, tissue kallikrein, tryptase and chymase are involved in extracellular matrix degradation, either by direct catalysis of matrix components, or by activating the various members of the MMP family (see Section 1.3.3)(Clark and Murphy 1999, and Chapman *et al.,* 1996).

1.3.2 Cathepsins

Cathepsins are papain family cysteine proteases, which are involved in a variety of physiological processes including proenzyme activation, enzyme inactivation, antigen presentation, hormone maturation, tissue remodelling and bone matrix resorption (Rochefort *et al.,* 2000, Linnervers *et al.,* 1997, Mort and Buttle 1997, and Fosang *et al.,* 1992). They are glycoproteins and all contain an essential cysteine residue in their active site, but family members differ in some enzymatic properties, including substrate specificities and pH stability (Linnevers *et al.,* 1997). The only members of the cathepsin family expressed in cartilage are cathepsins B and D (Mort and Buttle

1997, and Woessner 1973, respectively). Under acidic conditions cathepsin D cleaves aggrecan, and these cleavage sites within the aggrecan core protein have been shown to be utilised in cartilage exposed to acidic pH *in vitro* (Handley *et al., 2001*).

1.3.3 Matrix Metalloproteinases (MMPs) / Matrixins

Matrix Metalloproteinases are a family of zinc metallo-endopeptidases secreted by cells, and are responsible for much of the turnover of matrix components such as collagens and proteoglycans in normal embryogenesis and remodelling as well as in many diseases such as arthritis, cancer periodontis and osteoporosis (Davidson *et al.,* 1999, Herrera *et al.,* 2002, Nabeshima *et al.,* 2002, Vicenti *et al.,* 1996, and Yamada *et al.,* 2002). They are part of the "MB clan" of metalloproteinases, which contain the zinc-binding motif HEXXHXXGXXH at their active site. Members of the MB clan are generically referred to as 'Metzincins' as they all contain a conserved methionine that forms a turn eight residues downstream of the active site. The MB clan contains a number of families and MMPs are in family M10, which is further subdivided into A and B with MMPs being in subfamily A (also known as the matrixins).

Numbers identify MMPs from vertebrate species whereas those from invertebrates are only designated by trivial names. There are 25 human MMPs known at this point in time (Somerville *et al.,* 2003) with over 30 having been described in all species (see Table 1.3). All MMPs share a common catalytic core with a zinc molecule in the active site.

Most MMPs are produced as zymogens with the exceptions being the Membrane Type (MT)-MMPs (Cawston 1998), with a signal sequence and propeptide domain containing a conserved cysteine that chelates the active zinc (Van Wart and Birkedal-Hansen 1990). The zinc atom requires four ligands, the fourth of which is the cysteine conserved in the prodomain in the inactive (zymogen) form. In the active form of the enzymes this site is occupied by water. The generally conserved sequence around the chelating cysteine (PRCGVP) has been named the "cysteine switch". MMP-23 lacks this conserved cysteine and has a very different propeptide domain. MMP-26 contains a mutated cysteine switch, which has recently been shown to be inactive (Marchenko *et al.,* 2002). A subset of MMPs, including the membrane type MMPs (MMP-

14 [MT1-MMP], MMP-15 [MT2-MMP], MMP-16 [MT3-MMP], MMP-17 [MT4-MMP], MMP-24 [MT5-MMP] and MMP-25 [MT6-MMP]) as well as MMPs -11, -21, -23 and -28 contain a basic prohormone convertase cleavage sequence (RRKR, RRRR, RKRR etc.) which may be cleaved by members of the Golgi associated Paired basic Amino acid Cleaving Enzyme (PACE) / Furin family of enzymes resulting in the loss of a peptide of about 10kD (Pei and Weiss 1996) and secretion of the enzymes to the cell surface in an active form (Cawston 1998). Once activated MMPs can cleave other MMPs to activate them. This is especially so of the membrane type (MT)-MMPs that are located at the cell surface and are thought to activate proMMPs once they have left the cells interior (Knauper *et al.,* 2002, and d'Ortho *et al.,* 1998). Secreted proMMPs are activated *in vitro* by proteinases and non-proteolytic agents such as SH-reactive agents and reactive oxygen (Nagase and Woessner 1999).

The catalytic domain of the MMPs is around 170 amino acids in length and includes a zinc-binding motif of the sequence HEXXHXXGXXH (Nagase and Woessner 1999). In addition to the catalytic zinc the catalytic domain also contains a structural zinc and 2-3 calcium ions that are required for stability and catalytic activity (Nagase and Woessner 1999). The active site forms a long groove that divides the domain and in the zymogen form of the enzyme the propeptide domain lies in reverse orientation in this groove, with the cysteine switch in close proximity to the active site zinc molecule. The MMPs differ in the geography of the active site groove, allowing for different substrate and inhibitor specificities. Differences in the depth of the S1 pocket of some MMPs has allowed generation of synthetic inhibitors that selectively bind the MMPs and contain zinc chelators to inhibit the active site. The peptidomimetic MMP inhibitors are being used in a number of clinical trials to inhibit MMP function in different diseases (Porter *et al.,* 1994, Beckett *et al.,* 1996, Karran *et al.,* 1995, and Greenwald *et al.,* 1994).

All but two MMPs (MMP-7 and -26) contain a regulatory subunit, the haemopexin domain, at the carboxy-terminus separated from the catalytic domain by a variable hinge region (shown in Figure 1.7). The haemopexin domain is a four-blade propeller, with a calcium-binding site nestled in the folds (Gomis-Ruth *et al.,* 1996). Calcium seems to be required for some MMP-substrate interactions but not others. The haemopexin domain is in the vitronectin family and is known to bind heparin. Heparin has been shown to potentiate some MMP activities and MMPs are often found associated with heparan sulphate proteoglycans on the cell surface. The haemopexin domain is thought to confer much of the substrate specificity to the MMPs, and is involved in activation as well as inhibition of the MMPs (Tam *et al.,* 2002, and Lehti *et al.,* 2002). Final activation of MMPs often includes shedding of the haemopexin domain, and the isolated haemopexin domain has been shown to inhibit intact MMPs (Tam *et al.,* 2002). The hinge region also confers specificity to the MMPs, by setting the orientation of the haemopexin domain and the catalytic domain.

The membrane type MMPs (MMP-14 [MT1-MMP], MMP-15 [MT2-MMP], MMP-16 [MT3-MMP], MMP-17 [MT4-MMP], MMP-24 [MT5-MMP] and MMP-25 [MT6-MMP]) lack the carboxy-terminal haemopexin-like domain, but they do have a transmembrane domain that anchors them to the cell surface followed by a cytoplasmic domain (see Figure 1.7). The cytoplasmic domain is thought to be involved in cytoskeletal signalling cascades, and may be directly phosphorylated by various kinase cascades. MMP-17 (MT4-MMP) and MMP-25 (MT6-MMP) have no cytoplasmic domains and are thought to be GPI-anchored to the cell surface (Itoh *et al.,* 1999, and Kojima *et al.,* 2000).

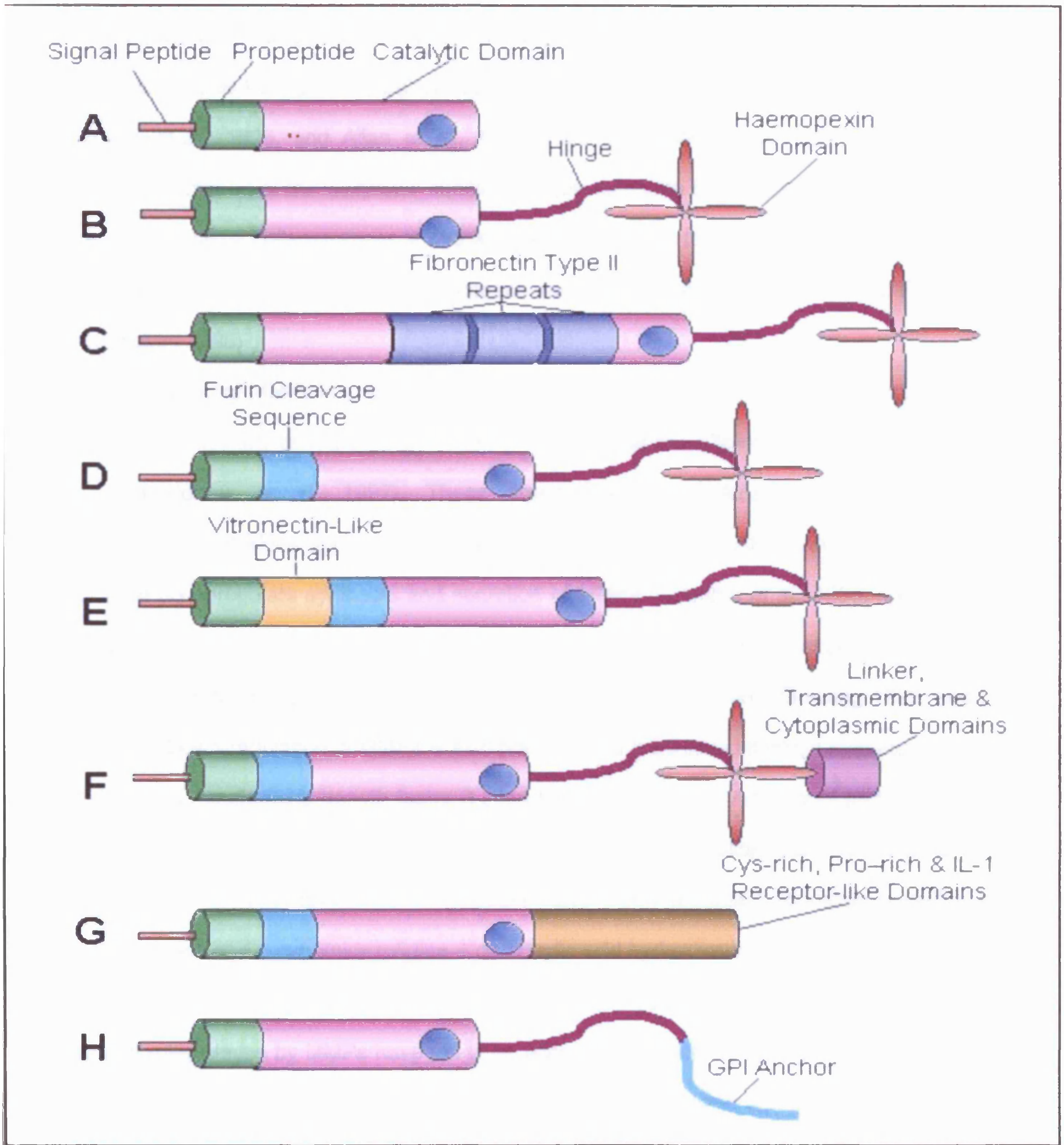

Figure 1.7 Domain organisation of zymogen forms of MMPs adapted from Alexander 2002

MMPs-2 and -9 are unique among MMPs in that three fibronectin type II domains are inserted in their catalytic domains in the vicinity of the active site (Briknarová *et al.,* 1999). These type II modules account for the affinity of MMP-2 for gelatin, type I and IV collagen, elastin and laminin (Bányai and Patthy 1991, Bányai *et al.,* 1994, Shipley *et al.,* 1996, Murphy *et al.,* 1994, Steffensen *et al.,* 1995, and Allan *et al.,* 1995). The fibronectin-like domain of MMP-9 is an important determinant of the enzyme's fibrillar collagen substrate specificity. It allows the enzyme to bind to and cleave collagen types V and XI, events which are thought to be involved in several normal physiological and pathological processes such as metastasis and arthritis (O'Farrell and Pourmotabbed 1998)

Expression of MMPs is turned on by a variety of agents acting through regulatory elements of the gene, particularly the Activating Protein (AP)-1 binding site (Goldring 1993, Cawston *et al.,* 1995, and Korzus *et al.,* 1997). These agents include cytokines and growth factors. Proinflammatory cytokines such as Interleukin (IL)-1α, Osmium Tetroxide and Tumour Necrosis Factor (TNF)-α upregulate synthesis and secretion of MMPs (Cawston *et al.,* 1998, and Koshy *et al.,* 2002). Some growth factors such as epidermal growth factor and fibroblast growth factor have also been shown to upregulate MMP production (Kondapaka *et al.,* 1997, Goldring 1993, and Liu *et al.,* 2002). Down-regulation of MMPs is also possible and is achieved through mediators such as IL-10, IL-4 and transforming growth factor ß (Van Roon *et al.,* 1996). A family of protein inhibitors of MMPs have been identified and termed Tissue Inhibitors of MetalloProteinases (TIMPs) (see Section 1.3.6). Many synthetic MMP inhibitors have been produced including arylsulphonyl hydroxamic acids and 2-oxo-imidazolidine-4-carboxylic acid hydroxamides (Baxter *et al.,* 2001, and Robinson *et al.,* 2001, respectively) but they inhibit a broad spectrum of MMPs and their effects on the normal roles played by MMPs have yet to be ascertained. Recently synthetic inhibitors specific for gelatinases (MMPs -2 and -9) having minimal cross activity to other types of MMPs have been produced (Bernado *et al.,* 2002)

Table 1.3 Matrix Metalloproteinases (MMPs) able to cleave components of the cartilage extracellular matrix, their alternative names and domain composition (adapted from Alexander 2002, and Somerville *et al.,* 2003)

MMP	PSEUDONYMS	DOMAIN ORGANISATION (FIGURE 1.7)	SUBSTRATES
MMP-1	Collagenase-1, Fibroblast Collagenase, Tissue Collagenase, Interstitial Collagenase	B	Collagens I, II, III, VII, VIII, X, Aggrecan, Gelatin, MMP-2, MMP-9
MMP-2	Type IV Collagenase, Gelatinase A, TBE-1	C	Collagens I, II, III, IV, V, VII, X, XI, Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-9, MMP-13
MMP-3	Procollagenase, PTR1 protein, Stromelysin-1, Transin-1	B	Collagens II, III, IV, IX, X, XI Aggrecan, Elastin, Fibronectin, Gelatin, Laminin. MMP-7, MMP-8, MMP-13
MMP-7	Matrilysin, Matrin, PUMP-1 Protease, Uterine Metalloproteinase	A	Collagens IV, X, Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-1, MMP-2, MMP-9
MMP-8	Collagenase-2, Neutrophil Collagenase, PMNL	B	Collagens I, II, III, V, VII, VIII, X, Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-9	Type IV Collagenase, Gelatinase B	C	Collagens IV, V, VII, X, XIV Aggrecan, Elastin, Fibronectin, Gelatin
MMP-10	Stromelysin-2, Transin-2	B	Collagens III, IV, V Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-1, MMP-8
MMP-11	Stromelysin-3	D	Aggrecan, Fibronectin, Laminin
MMP-12	HME, Macrophage Metalloelastase	B	Collagen IV, Elastin, Fibronectin, Gelatin, Laminin
MMP-13	Collagenase-3	B	Collagen I, II, III, IV, Aggrecan, Gelatin
MMP-14	MT1-MMP	F	Collagens I, II, III, Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-2, MMP-13
MMP-15	MT2-MMP	F	Fibronectin, Gelatin, Laminin, MMP-2
MMP-16	MT3-MMP	F	Collagens I,III, Gelatin, Aggrecan, Casein, Fibronectin, Laminin, Perlecan, Vitronectin
MMP-17	MT4-MMP	H	Fibrin, Gelatin

MMP-19	RASI-1	B	Collagen IV, Fibronectin, Aggrecan, COMP, Laminin, Gelatin
MMP-20	Enalysin	B	Aggrecan, Amelogenin, COMP
MMP-23	CA-MMP		Gelatin
MMP-24	MT5-MMP	F	Gelatin, Chondroitin Sulphate, Dermatan Sulphate, Fibronectin
MMP-25	MT6-MMP, Leukolysin	H	Collagen IV, Gelatin, Fibronectin, Laminin-1
MMP-26	Matrilysin-2, Endometase	A	Collagen IV, Gelatin, Fibronectin
MMP-28	Epilysin	D	Casein

MMPs play roles in normal tissue morphogenesis, wound healing, nerve growth, bone remodelling, and embryonic development (Nagase and Woessner 1999), but they also play roles in pathological processes such as arthritis, cancer etc., (Davidson *et al.,* 1999, Herrera *et al.,* 2002, Nabeshima *et al.,* 2002, Vicenti *et al.,* 1996, and Yamada *et al.,* 2002). For example in the later stages of cartilage degradation significant breakdown of type II collagen occurs through the action of collagenases (Hollander *et al.,* 1994) and this may represent irreversible cartilage damage (Cawston *et al.,* 1998).

1.3.5 A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS)

A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) are a family of extracellular proteases found in both mammals and invertebrates (see Table 1.4). First isolated in 1997 (Kuno *et al.,* 1997), these proteases are clearly of high biological relevance, with expression being upregulated in cancer and tissue inflammation. All members of the ADAMTS family have similar structures (see Figure 1.8).

Like the ADAM family proteins the ADAMTS start at their amino-termini with a signal sequence. This is followed by a putative prodomain which varies in length between 220-300 amino acids, except for ADAMTS-13 which has an unusually short prodomain of 74 amino acids (Cal *et al.,* 2002). Three cysteine residues are present within each prodomain, except in the case of ADAMTS-2, ADAMTS-3 and ADAMTS-14, which contain two and ADAMTS-13 that contains one (Cal *et al.,* 2002).

A furin cleavage site with the consensus sequence RX(K/R)R marks the end of the prodomain of all the mammalian ADAMTSs (Molloy *et al.,* 1992, Cal *et al.,* 2002, Llamazares *et al.,* 2003, Abbaszade *et al.,* 1999, Tortorella *et al.,* 1999, and Kuno *et al.,* 1997). ADAMTS-5 has three consensus sequences for Furin (Hurskainen *et al.,* 1999) or another prohormone convertase and ADAMTS-6 and ADAMTS-7 have one such site each (Hurskainen *et al.,* 1999). The most carboxy-terminal of the cleavage sites is likely to be the one utilised in the production of the mature active form of the protease. ADAMTS family members are also predicted to contain the cysteine switch described in members of the MMP family, although activation of ADAMTS proteins through a cysteine switch mechanism has not yet been described. It appears that the zymogen form of ADAMTS proteins resides intracellularly and that secreted enzyme is furin-processed (Apte 2004).

Following the prodomain is the ADAM metalloproteinase domain, which is well conserved among all members of the family (Bode *et al.,* 1993) The catalytic site consensus sequence HEXGHXXGXXHD is present in all ADAMTS and therefore all the proteins are presumed to be catalytically active (Molloy *et al.,* 1992, and Tang 2001). Five cysteine residues are upstream of the zinc binding sequence, while three are downstream, except in ADAMTS-2, -3 and -14 which each have three cysteines upstream and three downstream (Bode *et al.,* 1993). ADAMTS-13 is unique in having three cysteines upstream of the zinc binding sequence and two downstream (Cal *et al.,* 2002). In common with all MMPs and reprolysins the zinc-binding signature in ADAMTSs is followed by a methionine residue forming the "Met-turn", a tight turn arranged as a right-handed screw carboxy-terminal to the zinc-binding signature (Bode *et al.,* 1993).

Following the catalytic domain is the disintegrin-like domain, a region of 60-90 amino acids with 35-45% protein sequence homology to the snake venom metalloproteinases (Huang *et al.,* 1997). The disintegrin-like domain contains eight cysteines in all ADAMTSs except ADAMTS-6, which has six (Hurskainen *et al.,* 1999). Although the disintegrin domain is similar in its homology to the disintegrins this does not imply that it shares a similarity of function with these proteins.

A thrombospondin (TSP) motif homologous to the type-1 repeat of thrombospondins-1 and -2 follows the disintegrin-like domain (Bornstein 1992). The thrombospondin motifs distinguish the ADAMTSs from the ADAMs. The first TSP is very similar in all the ADAMTSs and is 48-54 amino acids long (Kuno *et al.,* 1997). The downstream TSPs are much more variable in sequence. In some of the ADAMTSs the TSP motifs contain a consensus binding site (BBXB) for GAG chains. The thrombospondin motifs of the ADAMTSs have been proposed to interact with heparin in a similar way to the thrombospondin motifs of thrombospondins-1 and -2 (Bornstein 1992).

The first central TSP motif is followed by a cysteine rich domain containing 10 conserved cysteine residues that is poorly conserved between members of the ADAMTS family. This domain is so-called to distinguish it from the cysteine free spacer domain that follows it. The amino-terminal portion of the spacer domain contains several hydrophobic amino acids that are well conserved whereas the carboxy-terminal portion of the spacer domain is extremely variable.

A second set of TSP repeats follow the spacer domain and vary hugely in number between members of the ADAMTS family. ADAMTS-4 does not have any TSP repeats at this position with its carboxy-terminus being its spacer domain (Tortorella *et al.,* 1999). In ADAMTS-9 and 20 there are short linker peptides which space the TSPs (Cal *et al.,* 2002). In ADAMTS-12 a second long cysteine free spacer domain separates the two sets of TSP repeats forming a distinctive double array (Cal *et al.,* 2002).

The final domain of the ADAMTS family members may be formed by one of four so called "carboxy-terminal modules". ADAMTS-9 and -20 contain a module only otherwise found in their ortholog Gon-1 (Llamazares *et al.,* 2003). A number of ADAMTSs including ADAMTS-10 and -19 contain a Protease LACunin (PLAC) domain whereas ADAMTS-2, -3 and -14 (the procollagen propeptidases) all contain a unique carboxy-terminal domain in which a PLAC domain is embedded (Cal *et al.,* 2002). ADAMTS-13 is the only member of this family to contain Complement Cla/Clr, sea urchin Uegf protein, Bone morphogenetic protein 1(CUB) domains. There are two located at the carboxy-terminus (Cal *et al.,* 2002).

An ADAMTS-like protein (ADAMTS-L) has recently been identified and cloned (Hirohata *et al.,* 2002). It is composed of thrombospondin repeats with a spacer and cysteine rich region between them. The role of this protein has yet to be elucidated.

Table 1.4 Mammalian members of the ADAMTS family their pseudonyms, chromosomal location, sites of expression and postulated functions (Arner 2002, Tang 2001, Tang and Hong 1999, Cal *et al.,* 2002, Vazquez *et al.,* 1999, Hurskainen *et al.*, 1999, and Llamazares *et al.*, 2003)

ADAMTS	PSEUDONYMS	HUMAN CHROMOSOMAL LOCATION	FUNCTION	SITES OF EXPRESSION
ADAMTS-1	METH-1, KIA1356	21q21-q22	Inflammatory response, angiogenesis, organ morphogenesis	Embryonic lung, liver and kidney, adult cartilage,
ADAMTS-2	Procollagen N-proteinase	5q23-q24	Procollagen processing	
ADAMTS-3	KIAA0366	4q21		
ADAMTS-4	Aggrecanase-1, KIAA0688	1q21-q23	Aggrecan cleavage, Brevican cleavage	Synovium, cartilage, brain, placenta
ADAMTS-5	Aggrecanase-2, ADAMTS11	21q22.1-q22	Aggrecan cleavage	Cartilage, brain, placenta
ADAMTS-6		5q13		
ADAMTS-7		15pter-qter		
ADAMTS-8	METH2	11q25	Angiogenesis	
ADAMTS-9	KIAA1312	3p14.2		
ADAMTS-10		19p13.3		
ADAMTS-11	Also known as ADAMTS-5			
ADAMTS-12		5q35	May play roles in pulmonary cells during foetal development or in tumour processes	
ADAMTS-13		9q34		Foetal liver, adult prostate and brain, melanoma and colon carcinoma cells
ADAMTS-14		10q 21.3	May cleave type II procollagen in the absence of ADAMTS-2	Foetal lung and kidney
ADAMTS-15		11q25		Foetal kidney and Liver
ADAMTS-16		5p15		Foetal lung and kidney, adult brain
ADAMTS-17		15q24		Foetal lung, adult prostate and brain
ADAMTS-18		16q23		Foetal lung and liver, adult prostate and brain
ADAMTS-19		5q31		Foetal lung, osteosarcoma cell lines
ADAMTS-20				Testis and brain

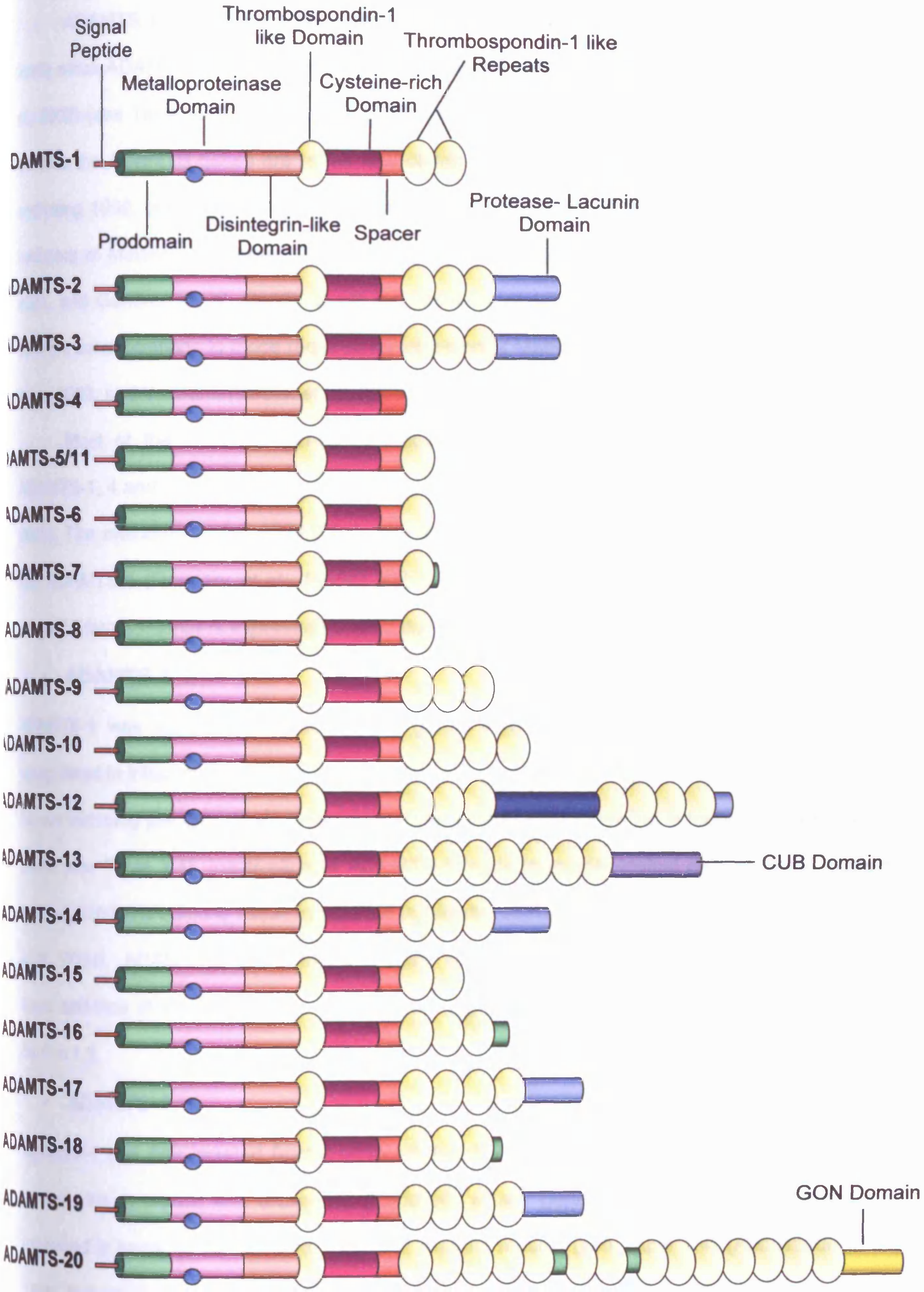

Figure 1.8 Predicted protein structures of members of the ADAMTS family in their zymogen form (adapted from Cal *et al.*, 2002, and Llamazares *et al.*, 2003).

ADAMTS knockout mice have shown the possibility of redundancy within this protease family since ADAMTS-14 may cleave type II procollagen in the absence of ADAMTS-2 (Colige *et al.*, 2002) (see Table 1.4). Unlike several of the MMP genes that are clustered in the mammalian genome the ADAMTS genes are rather well distributed amongst the human chromosomes (Tang and Hong 1999, and Tang 2001). Some members of the ADAMTS family are inhibited by Tissue Inhibitors of Metalloproteinases (TIMPs), which are discussed in Section 1.3.6 (Kashiwagi *et al.*, 2001, and Gendron *et al.*, 2003). Synthetic inhibitors of MMPs also inhibit ADAMTS activity at different concentrations to those required for inhibition of MMP activity (Little *et al.*, 2002a, Cherney *et al.*, 2003, and Vankemmelbeke *et al.*, 2003).

Most of the ADAMTS genes are expressed at low levels in adult tissues. However ADAMTS-1, 4 and 7 are relatively more abundant and ubiquitous (Tang and Hong 1999, and Tang 2001). The members of the ADAMTS family expressed in cartilage are ADAMTS-1, ADAMTS-4 and ADAMTS-5 (Flannery *et al.*, 1999b), although their expression is not cartilage specific. The ADAMTS family members expressed in articular cartilage are discussed below.

- **ADAMTS-1**

ADAMTS-1 was identified in a screen for genes involved in cancer cachexia and is highly upregulated in inflammation (Kuno *et al.*, 1997). It is expressed at high levels in a variety of foetal tissues including placenta, brain, heart, lung, liver, spleen and kidney but is found at lower levels in adults (see Table 1.4). Inactivation of ADAMTS-1 leads to morphological defects in the kidneys, adrenal gland and adipose tissue in addition to growth retardation and infertility in females (Shelley *et al.*, 2002). ADAMTS-1 can cleave aggrecan, versican and brevican although the relevance of these activities *in vivo* is unclear. Cleavage of aggrecan by ADAMTS-1 is further discussed in Section 1.5.

- **ADAMTS-4**

ADAMTS-4 was first isolated from Interleukin (IL)-1 stimulated bovine nasal cartilage, and the human ortholog cloned and expressed (Tortorella *et al.*, 1999) It has since been shown to be expressed in heart, brain, placenta, lung and skeletal muscle (see Table 1.4) (Abbaszade *et al.*, 1999). Full length active human ADAMTS-4 has a predicted molecular weight of ~68kD and has been shown to undergo autocatalytic carboxy-terminal truncation to generate two discrete isoforms of 53 and 40kD (Flannery *et al.*, 2002). The role of ADAMTS-4 in aggrecan cleavage during disease states such as arthritis is discussed in Section 1.5.

- **ADAMTS-5**

ADAMTS-5 was first isolated from IL-1 stimulated bovine nasal cartilage and the human ortholog sequenced and cloned (Abbaszade *et al.*, 1999). At this time it was designated ADAMTS-11, however the protein had already been isolated, cloned and designated ADAMTS-5. When the sequences for ADAMTS-5 and ADAMTS-11 were shown to be orthologs of each other the protein was officially designated ADAMTS-5. ADAMTS-5 has since been shown to be highly expressed in placenta with lower levels in heart, liver and brain (Abbaszade *et al.*, 1999). ADAMTS-5 levels during endochondral ossification increased during the hypertrophic stage (Makihira *et al.*, 2003). This implies a role for ADAMTS-5 in aggrecan degradation during endochondral ossification. In growth plate cartilage the enzyme was also shown to be upregulated by thyroid hormone (Makihira *et al.*, 2003). The role of ADAMTS-5 in aggrecan cleavage during disease states such as arthritis is discussed in Section 1.5.

1.3.6 Tissue Inhibitors of MetalloProteinases (TIMPs)

The family of Tissue Inhibitors of MetalloProteinases (TIMPs) currently comprises 4 members in humans: TIMP-1, -2, -3 and -4. These are homologous in sequence and have similar secondary and tertiary structures including 6 well conserved disulphide bonds. Mammalian TIMPs are two-domain molecules, having an amino-terminal domain of ~125 amino acids and a smaller carboxy-terminal domain of ~65 residues. Each domain is stabilised by three disulphide bonds (Williamson *et al.*, 1990).

Inhibition studies with recombinant TIMPs have shown them to bind to MMPs with varying affinity implicating distinct functions in vivo (Brew *et al.*, 2000, and Woessner 2002). Structural and functional studies of TIMP-1 and -2 (Murphy *et al.*, 1991, Huang *et al.*, 1997, Williamson *et al.*, 1997, and Gomis-Rüth 1997) have shown their inhibitory activity to reside in their amino-terminal domain.

In addition to the inhibitory activity of TIMPs, some are also involved in activation of MMPs e.g. proMMP-2 is proposed to interact via its hemopexin domain with the carboxy-terminal regions of TIMPs -2, -3 and -4 (Overall *et al.,* 1999, Bigg *et al.,* 1997 and Butler *et al.,* 1998). Each of the TIMPs is bound to the cell surface by MT1-MMP. Another molecule of MT1-MMP must be present near by on the cell surface to activate the proMMP-2 since the MT1-MMP molecule bound to the TIMP is also inhibited by them (Strongin *et al.,* 1995, and Butler *et al.,* 1998). The association of two or more molecules of MT1-MMP in this way was recently shown by interactions of their hemopexin domains (Itoh *et al.,* 2001). A similar activation has been suggested for proMMP-9 via interaction with TIMPs -1 and -3 (Butler *et al.,* 1998, and Goldberg *et al.,* 1992).

In addition to their interactions with MMPs TIMPs also inhibit other enzymes including members of the ADAM family (see Section 1.3.4). Inhibition of ADAM-10 by TIMP-3 has been shown to prevent shedding of cell surface anchored tumour necrosis factor (TNF)-α receptor (Smith *et al.,* 1997), IL-6 receptor (Hargreaves *et al.,* 1998) and syndecans -1 and -4 (Fitzgerald *et al.,* 2000). Direct evidence of TIMP-3's ability to inhibit ADAM-17 and -10 has been established (Amour *et al.,* 1998, and Hurskainen *et al.,* 1999). More recently ADAM-10 has been shown to be inhibited by TIMP-1 and TIMP-3 whereas ADAM-17 is only inhibited by TIMP-3 (Amour *et al.,* 2000).

TIMP-1 inhibits most MMPs with K_i levels of 0.1 - 2.8nM (Murphy and Willenbrock 1995). TIMP-1 has a higher affinity for full length MMP-1 as compared with MMP-1 that lacks the carboxy-terminal hemopexin domain (Taylor *et al.,* 1996). The removal of the hemopexin domain from MMPs often results in an approximately 5-fold to 20-fold increase in the K_i value, indicating that the hemopexin domain assists the interaction of TIMP-1 with MMPs (Nagase and Brew 2002).

TIMPs -2, -3 and -4 inhibit all MMPs so far tested. TIMP-2 binds to MMP-2 most tightly with the dissociation constant being such that the interaction is essentially irreversible (Hutton *et al.,* 1998). The tight nature of this interaction is largely due to the carboxy-terminal domain of TIMP-2 and the carboxy-terminal domain of MMP-2 (Willenbrock *et al.,* 1993). TIMP-3 binds to MMP-3 with relatively low affinity (K_i = 67nM) (Kashiwagi *et al.,* 2001).

TIMP-3 has several properties distinct from those of other TIMPs, which include its ability to bind tightly to the extracellular matrix (Pavloff *et al.*, 1992 and Yu *et al.*, 2000), apoptotic effects on a number of cell types (Smith *et al.*, 1997, and Baker *et al.*, 1998) and inhibition of TACE (Smith *et al.*, 1997). TIMP-3 was first isolated as a 21kD protein secreted from chick fibroblasts transformed with Rous sarcoma virus and TIMP-3 has been localised to human chromosome 22 (Apte *et al.*, 1994) and binds via interaction of its amino-terminal domain to polyanionic components of the extracellular matrix (Yu *et al.*, 2000). The amino-terminal domain of TIMP-3 has been shown to inhibit the catalytic activity of both ADAMTS-4 and -5 (see Sections 1.3.5 and 1.5.2) (Kashiwagi *et al.*, 2001). It has since been shown that this interaction is not unique to TIMP-3 as TIMPs-1, -2 and -4 also bind to ADAMTS-4, but with much lower affinity than TIMP-3 (Hashimoto *et al.*, 2001). TIMP-3 complexes have been pinpointed as causes of Sorsby's fundus dystrophy an autosomal dominant inherited retinal degenerative disease that leads to blindness (Yeow *et al.*, 2002).

1.4 Articular Cartilage Disease States

1.4.1 Osteoarthritis

More than 2 million people visited their GP in the past year because of osteoarthritis (Arthritis Research Campaign 2005). At least 4.4 million people in the UK have X-ray evidence severe osteoarthritis in their knees (Arthritis Research Campaign 2005). Clinically osteoarthritis is characterised by joint pain, tenderness, limitation of movement, occasional effusion and variable degrees of inflammation without systemic effects (Kuettner and Goldberg 1995).

In the early stages of the disease the surface of cartilage, or even synovium in some individuals, becomes inflamed and swollen. There is a loss of proteoglycan molecules and other tissue components that results in loss of the water they entrapped within the matrix. Fissures and pits appear in the cartilage. As the disease progresses and more tissue is lost, the cartilage loses elasticity and fluid. It becomes increasingly prone to damage due to repetitive use and injury. Eventually large amounts of cartilage are destroyed, leaving the ends of the bone within the joint unprotected.

The biological factors leading to the deterioration of cartilage in osteoarthritis are not entirely understood. One view of osteoarthritis is as a failure to maintain a balance between synthesis and degradation of matrix components. Synthesis of cartilage components appears to be dependent on a number of growth factors including insulin-like growth factor (IGF)-1 and Transforming Growth Factor (TGF)β. Despite the fact that IGF-1 has been shown to reduce the development of osteoarthritis in animal models (Rogachefsky *et al.*, 1993), the evidence for its role in humans is conflicting, with increased (Dore *et al.*, 1995), decreased and normal concentrations (Pagura *et al.*, 2004) being detected in patients with osteoarthritis. The picture is equally confusing with TGFβ (Creamer and Hochberg 1997). Degradative enzymes such as MMPs (see Section 1.3.3) are found in increased concentrations in osteoarthritic cartilage, and their synthesis by chondrocytes can be stimulated by Interleukin-1α (Woessner 1994). Blockage of MMPs by doxycycline, in animal models, can reduce the severity of osteoarthritis lesions (Ryan *et al.*, 1996). Other enzymes involved in the degradation of extracellular matrix components in osteoarthritis include members of the ADAMTS family (see Section 1.3.5) (Sandy *et al.*, 1991a, Ilic *et al.*, 1992, and Loulakis *et al.*, 1992).

Subchondral bone changes are often seen on radiographs in patients with established osteoarthritis and increasingly these are viewed as an important cause of osteoarthritis, rather than the follow-on from cartilage damage.

Current treatment of osteoarthritis is purely to control symptoms because as yet there are no disease-modifying osteoarthritis drugs. The principal treatments in use are forms of pain control. Systematic reviews of both non-pharmacological therapies (e.g. exercise) and pharmacological therapies such as paracetemol and Non-steroidal Anti-Inflammatory Drugs (NSAID)(e.g. Aspirin) have been published (Towheed and Hochberg 1997a, Towheed and Hochberg 1997b and Puett and Griffin 1994).

1.4.2 Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune disease where an unknown environmental agent such as a virus or toxin triggers an autoimmune response in genetically susceptible individuals (Schiff 2000). Factors increasing an individual's susceptibility to rheumatoid arthritis include expression of the Human Leukocyte Antigen class II locus (Feldmann *et al.,* 1996). Internationally around 1% of individuals are diagnosed with rheumatoid arthritis, with 10-20% of the patients going on to develop a permanent disability (Tsou *et al.,* 2004).

It is generally accepted that the initial events in the development of rheumatoid arthritis is the proliferation of synovial cells with inflammation in the stroma of the synovial tissue (Zvaifler 1983). The pathology of rheumatoid arthritis extends throughout the synovial joint with even the normally acellular synovial fluid becoming enriched with neutrophils and macrophages (Feldmann *et al.,* 1996). The synovial membrane undergoes an increase in vascularity and infiltration of inflammatory cells, CD4+ T cells (Choy and Panayi 2001), macrophages, fibroblasts, mast cells (Bromley *et al.,* 1984), polymorphonuclear leukocytes (Mohr and Menninger 1980), and displaced (probably dedifferentiated) chondrocytes (Allard *et al.,* 1987).

At the junction of the synovial lining and the joint capsule a major source of tissue damage, termed the pannus, originates. This will eventually grow not only over the cartilage, but also into it, destroying the cartilage in the process (Tsou *et al.,* 2004). Cells both within the synovial lining and the pannus secrete inflammatory cytokines (e.g. Interleukin-1 and Tumour Necrosis Factor α) (Chu *et al.,* 1992) as well as MMPs (Choi and Panayi 2001).

Interleukin-1 and -6, and Tumour Necrosis Factor α are the important cytokines that drive inflammation in rheumatoid arthritis (Kumar *et al.,* 2001). There are many proteinases involved in rheumatoid arthritis that are produced by different cell types. MMPs are primarily produced by synovial fibroblasts and chondrocytes (Birkedal-Hansen *et al.,* 1993, and Nagase *et al.,* 1992). Interleukin-1α and Tumour Necrosis Factor α are able to stimulate the expression of adhesion molecules that promote the recruitment of inflammatory cells into the joint. This includes neutrophils that, once in a joint, release elastase and proteases that degrade the superficial zone of the cartilage (Moore and Dorner 1993). Biologic disease modifying anti-rheumatic drugs have been developed that antagonise the actions of Interleukin-1 and Tumour Necrosis Factor α (Olsen and Stein 2004, and O'Dell 2004).

1.5 Aggrecan Degradation in Health and Disease

One of the first pathological features of joint degeneration is loss of the cartilage proteoglycan aggrecan. This precedes both overt collagen catabolism and joint erosion by some time. Following joint injury and subsequent joint disease there is a loss of aggrecan metabolites from the cartilage matrix into the synovial fluid (Lohmander 1991). In the later stages of arthritis significant breakdown of type II collagen occurs (Hollander *et al.,* 1994). This is thought to represent irreversible cartilage damage (Cawston *et al.,* 1998).

1.5.1 Model Systems of Cartilage Aggrecan Degradation

Culture systems allow analysis of the metabolism of chondrocytes and their extracellular matrix. There are several culture systems in use; cartilage explants, isolated chondrocyte monolayer cultures, cultures of isolated chondrocytes suspended in agarose or alginate, and pellet cultures.

Articular cartilage explants and cultures of isolated chondrocytes in agarose gel offer numerous advantages over those grown in monolayer culture where chondrocytes are prone to dedifferentiation as the forces acting on the chondrocyte through the matrix have been removed. The pressure of the matrix on the chondrocytes and its relatively poor perfusion of nutrients, in mature cartilage, may contribute to maintaining the phenotype of the chondrocyte. Spirito *et al.*, reported that the phenotype of bovine chondrocytes was maintained for longer in chondrocyte-agarose cultures than parallel monolayer chondrocyte cultures (Spirito *et al.,* 1993).

A number of studies using chondrocytes embedded in agarose as a model system to investigate the effect of exogenous agents (such as TIMPs-1 and -2 and polysulphated polysaccharides) on GAG release and chondrocyte phenotype have been published (Kuroki *et al.*, 2003, Verbruggen *et al.,* 1999, and Verbruggen *et al.,* 2000). Researchers have also used chondrocyte-agarose cultures as a model system for the investigation of effects of hydrostatic pressure on the structure and composition of the extracellular matrix secreted by chondrocytes

(Toyoda *et al.,* 2003a, Kelly *et al.,* 2004, Quinn *et al.,* 2002, Mauck *et al.,* 2002, and Toyoda *et al.,* 2003b).

All culture systems used to study chondrocyte / matrix metabolism may be exposed to catabolic stimulants, mechanical loading or enzyme inhibitors and drugs. In cultures where no secreted matrix is present the effects of these conditions on the chondrocytes themselves may be investigated. Long term culture of chondrocytes embedded in agarose allows an extracellular matrix to be established around the chondrocytes, restoring the physical pressures exerted on the cells by the cartilage extracellular matrix. This matrix is almost free of the metabolites seen in explant matrices. Hence, the effects of catabolic stimulants, mechanical loading or enzyme inhibitors and drugs can be examined in an intact matrix without the complication of already present cartilage metabolites.

Pioneering the chondrocyte-agarose model system Aydelotte and Kuettner 1988 allowed bovine articular chondrocytes suspended in agarose to secrete a matrix, before the addition of catabolic enhancers such as IL-1 and retinoic acid to the culture medium. The morphology of the matrix was investigated using antibodies to cartilage proteoglycans before and after treatment with these catabolic enhancers. The results showed diminished proteoglycan synthesis and enhanced proteoglycan catabolism in the cultures treated with IL-1 and retinoic acid compared to controls. A more recent publication showed IL-1β to suppress aggrecan synthesis in human articular chondrocytes embedded in agarose (Wang *et al.,* 2001).

One of the major problems with agarose cultures is the difficulty of retrieving the cells, however a thermosensitive gel culture system has recently been described by An *et al.,* 2001. The polymer described is a copolymer of poly (N-isopropylacrylamide) and acrylic acid and has the ability to polymerise at temperatures of 37 °C and over and liquefy when the temperature falls below 37 °C. This new polymer allows recovery of over 90% of the cells.

Cartilage explant culture systems allow *ex-vivo* analysis of the metabolism of chondrocytes and their extracellular matrix. Extracellular matrix macromolecules and chondrocytes are arranged as they were *in vivo* so allowing analysis of their metabolism in a 'native' matrix. Alternative systems for culturing chondrocytes change the chondrocyte phenotype with respect to expression of the enzymes potentially involved in cartilage degeneration (Flannery *et al.,* 1999c).

Little et al., used cartilage explant cultures from a variety of species to investigate aggrecan and link protein catabolism, showing 'aggrecanases' to perform the primary cleavage in both untreated and IL-1 stimulated explants (Little *et al.,* 1999).

Cartilage explant cultures may be supplemented with enzyme inhibitors and other potential drugs to determine their effects on 'native' articular cartilage. Mason and Goh exposed bovine articular cartilage explants to sodium iodoacetate (a SH-dependent enzyme inhibitor). This was shown to inhibit proteoglycan synthesis and lactate production (Mason and Goh 1991).

However, in cartilage explant cultures the effects of such mediators may be obscured by the presence of matrix metabolites in the cartilage explant prior to its excision from the joint. In contrast the matrix secreted by chondrocytes embedded in agarose is almost free of the metabolites seen in explant matrices.

1.5.2 Degradation of Aggrecan *in Vivo* and *in Vitro*

For many years the degradation of aggrecan was thought to be carried out by MMPs. However, in 1991 Maniglia *et al.,* reported a series of amino-terminal amino acid sequences on aggrecan catabolites that were released from cartilage explant cultures exposed to IL-1α. The existence of these novel amino-terminal peptide sequences was soon confirmed by others (Sandy *et al.,* 1991a, Ilic *et al.,* 1992, and Loulakis *et al.,* 1992). These amino-terminal amino acid sequences did not correspond to previously published cleavage sites for any known matrix protease including the MMPs (Fosang *et al.,* 1991, 1992 and 1996). The term 'aggrecanase' was used to describe the unknown proteolytic enzyme able to cleave aggrecan at these sites (Hardingham and Fosang 1995). The major cleavage site described as an 'aggrecanase site' was located within the interglobular domain (IGD) of the aggrecan core protein at the Glu^{373}-Ala^{374} bond (Sandy *et al.,* 1991a and b). The main aggrecan catabolite, found in samples of synovial fluid from patients with arthritis, and released from cartilage explant cultures exposed to IL-1, both had the amino-terminal amino acid sequence ^{374}ARGSV... (human sequence enumeration) corresponding to cleavage at the 'aggrecanase site' within the IGD of aggrecan (Sandy *et al.,* 1992, and Lohmander *et al.,* 1993).

Aggrecan fragments have been identified in human articular cartilage extracts (Flannery *et al.,* 1992) and synovial fluids (Fosang *et al.,* 1995) that have been cleaved at the MMP susceptible Asn^{341}-Phe^{342} bond located within the interglobular domain (IGD). Recent *in vitro* studies have indicated that the primary cleavage of aggrecan occurs at the Glu^{373}-Ala^{374} bond, the so-called 'aggrecanase site', within the IGD of the aggrecan core protein during cartilage degradation and that cleavage by MMPs at the Asn^{341} -Phe^{342} bond may be a later event (Little *et al.,* 1999, and Van Meurs *et al.,* 1999).

Two 'aggrecanases' have so far been purified and cloned (Tortorella *et al.,* 1999, and Abbaszade *et al.,* 1999), they were both found to be members of the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, ADAMTS-4 (Aggrecanase-1) and ADAMTS-5 (Aggrecanase-2). Both have similar specificity for the Glu^{373}-Ala^{374} bond within the interglobular domain of the aggrecan core protein (Tortorella *et al.,* 1999).

Using an *in vitro* model of cartilage degradation it has been shown that ADAMTS-4 and -5 are the enzymes responsible for the loss of aggrecan from explant cultures of articular cartilage stimulated with IL-1α or TNFα (Tortorella *et al.,* 2001). Immunodepletion of media taken from IL-1α stimulated cartilage with an anti-ADAMTS-4 antibody led to a 75% reduction in 'IGD aggrecanase activity', whilst immunoprecipitation with an anti-ADAMTS-5 antibody led to a 15% decrease in 'IGD aggrecanase activity' (Tortorella *et al.,* 2001).

In addition to cleavage at the Glu^{373}-Ala^{374} bond, ADAMTS-4 and -5 have been shown to cleave at four sites within the chondroitin sulphate rich region of the aggrecan core protein, between globular domains G2 and G3 (Tortorella *et al.,* 2000b). These cleavages are at ...$GELE^{1480}$-$^{1481}GRGT$..., ...$KEEE^{1667}$-$^{1668}GLGS$..., ...$TAQE^{1771}$-$^{1772}AGEG$... and ...$VSQE^{1871}$-$^{1872}LGQR$.... ADAMTS-5 has also been shown to utilise a fifth cleavage site in the region spanning residues Gly^{1481} and ^{1667}Glu (Tortorella *et al.,* 2002). It has been suggested that these cleavages within the carboxy-terminal chondroitin sulphate binding domains of aggrecan occur more efficiently than cleavage within the interglobular domain at the Glu^{373}-Ala^{374} bond (Tortorella *et al.,* 2000b). ADAMTS-5 cleaves aggrecan approximately 2-fold slower than ADAMTS-4 (Tortorella *et al.*, 2002).

Binding-competition experiments conducted using native and deglycosylated aggrecan provided evidence for interaction of the cysteine rich and spacer domains of ADAMTS-4 with the GAG chains of aggrecan (Flannery *et al.*, 2002). This interaction between enzyme and substrate may facilitate cleavage of the aggrecan core protein by ADAMTS-4, suggesting that the GAG chains of aggrecan may be necessary for efficient ADAMTS-4 cleavage. 'IGD aggrecanase activity' has been shown to be inhibited by exogenous chondroitin sulphate or heparin (Sugimoto *et al.*, 1999). This suggests that these GAGs may compete for the binding of ADAMTS-4 to its aggrecan substrate.

Full length Furin-activated ADAMTS-4 has a predicted molecular weight of ~68kD and has been shown to undergo autocatalytic carboxy-terminal truncation to generate two discrete isoforms of 53 and 40kD (Flannery *et al.*, 2002). These smaller isoforms have a reduced affinity of binding to sulphated GAGs. Carboxy-terminal sequencing and mass analysis revealed that the GAG binding thrombospondin type I motif was retained following autocatalysis, indicating that sites present in the carboxy-terminal cysteine rich and / or spacer domains also effect binding of full length ADAMTS-4 to sulphated GAGs (Flannery *et al.*, 2002). It has recently been reported that carboxy-terminal truncation enhances the 'IGD aggrecanase activity' of ADAMTS-4, thus implying a potential regulatory function for the domains of the ADAMTS-4 carboxy-terminal region (Gao *et al.*, 2002).

Recent data indicates ADAMTS-4 to be constitutively expressed in chondrocyte monolayers and cartilage explants from both bovine nasal and articular cartilage, and that stimulation with IL-1 results in 'aggrecanase' activation (Pratta *et al.*, 2003). Investigation of a series of ADAMTS-4 deletion mutants has shown full length ADAMTS-4 to be the most effective enzyme for aggrecan degradation as measured by GAG release (Kashiwagi *et al.*, 2004). However, interestingly, activity at the Glu^{373} - Ala^{374} bond within the interglobular domain of aggrecan was increased in the deletion mutants lacking the cysteine rich and spacer domains (Kashiwagi *et al.*, 2004). Treatment of porcine articular cartilage explants with IL-1α induced secretion of ADAMTS-4 isoforms of 46, 40 and 37kD, indicating carboxy-terminal truncation of ADAMTS-4 to have occurred (Kashiwagi *et al.,* 2004). These lower molecular weight ADAMTS-4 isoforms are predicted to have enhanced proteolytic activity along with decreased substrate specificity due to the loss of the carboxy-terminal GAG binding regions (Kashiwagi *et al.,* 2004). This suggests that ADAMTS-4 may digest other cartilage proteins as well as aggrecan when it is present in carboxy-terminally truncated isoforms.

Recently published data indicated ADAMTS-4 to bind to the carboxy-terminal region of fibronectin (see Section 1.2.3.1) and that this interaction inhibits the enzymes activity against aggrecan (Hashimoto *et al.,* 2004). However, the physiological relevance of this is not yet known.

ADAMTS-1 has also been shown to be able to cleave at the 'IGD aggrecanase site' *in vitro* (Kuno *et al.,* 2000, and Rodriguez-Manzaneque *et al.,* 2002), but only at supraphysiological concentrations and therefore its role *in vivo* has yet to be confirmed (Sandy and Verscharen 2001). The thrombospondin type I motifs of ADAMTS-1 bind to sulphated GAGs (Kuno and Matsushima 1998) and, thus may serve to influence its substrate specificity. Along with ADAMTS-12, ADAMTS-1 can undergo proteolytic removal of its carboxy-terminal region resulting in removal of domains that can bind to sulphated GAGs (Rodriguez-Manzaneque *et al.,* 2000, Cal *et al.,* 2001, and Wei *et al.,* 2002).

Since the roles of the three aggrecanases (ADAMTS-1, -4 and –5) in cartilage degradation have yet to be confirmed the term aggrecanase is used to refer to the enzymatic activity capable of cleaving the Glu^{373}-Ala^{374} bond within the interglobular domain of aggrecan.

'IGD aggrecanase activity' has been identified associated with chondrocyte membranes (Billington *et al.,* 1998). Here a combination of IL-1α and oncostatin M was used to stimulate the chondrocytes before membrane purification. An enzyme activity associated with the membranes was able to cleave aggrecan at the Glu^{373}-Ala^{374} bond. 'IGD aggrecanase activity' has been shown to associate with the extracellular matrix (Kuno and Matsushima 1998), but has also been shown to be a soluble activity (Hughes *et al.,* 1998).

The functions of aggrecanases in organs other than cartilage require further investigation. Their activity has been shown to be soluble (Ilic *et al.,* 2000). Unlike in cartilage, the activity of the enzymes in synovium, joint capsule (Ilic *et al.,* 2000) and tendon (Rees *et al.,* 2000) was not dependent on stimulation of the tissue with catabolic stimuli (e.g. IL-1 or TNF). Aggrecanases have also been shown to cleave versican and brevican (Rauch *et al.,* 1991, Matthews *et al.,* 2000, Sandy *et al.,* 2001, and Yamada *et al.,* 1995).

1.6 Aims of the Project

A primary event in the destruction of cartilage in arthritic diseases is the loss of aggrecan from the extracellular matrix of articular cartilage. During aggrecan breakdown important cleavage sites are utilised which reside within the interglobular domain (IGD) of the aggrecan core protein. The Asn^{341}-Phe^{342} bond is cleaved by members of the MMP family, whereas the second of the two cleavage sites, the Glu^{373}-Ala^{374} bond (also known as the IGD aggrecanase site) is cleaved by members of the ADAMTS family. Both ADAMTS-4 and -5 have been identified, cloned, and shown to readily cleave aggrecan at the IGD aggrecanase site (Tortorella *et al.,* 1999, Abbaszade *et al.,* 1999, and Sandy *et al.,* 2000). ADAMTS-1 has also been shown to be able to cleave at the IGD aggrecanase site *in vitro* (Kuno *et al.,* 2000, and Rodriguez-Manzaneque *et al.,* 2002), but only at supraphysiological concentrations and therefore its role *in vivo* has yet to be confirmed (Sandy and Verscharen 2001). ADAMTS-4 and -5 also cleave at a number of other sites along the length of the aggrecan core protein (Tortorella *et al.,* 2000a). 'IGD aggrecanase activity' has been shown to be membrane associated (Hascall *et al.,* 1999) but it has also been detected in media samples from chondrocytes embedded in agarose showing it to be soluble (Hughes *et al.,* 1997).

The model system used for these investigations is that of chondrocytes embedded in agarose. This system was pioneered by Aydelotte *et al.,* 1988 and allows analysis of the metabolism and catabolism of chondrocytes and their extracellular matrix.

TIMP-3 has several properties distinct from those of other TIMPs, which include its ability to bind tightly to the extracellular matrix (Pavloff *et al.,* 1992 and Yu *et al.,* 2000). The amino-terminal domain of TIMP-3 has been shown to inhibit the catalytic activity of both ADAMTS-4 and -5 (Kashiwagi *et al.,* 2001).

Therefore the aims of this project are:

- Investigate the aggrecan content of the extracellular matrix secreted in the model system of chondrocytes embedded in agarose.
- Determine the effects of exposure of the aggrecan present in the extracellular matrix of chondrocyte-agarose cultures to catabolic stimuli (IL-1α).

- Examine the secretion, sequestration and activation of the aggrecanases (ADAMTS-4 and -5) in this culture system using a series of mono- and polyclonal antibodies recognising various domains of the enzymes.
- Scrutinize the effect of TIMP-3 on the ADAMTS-4 and -5 present in the culture system.

Chapter 2: General Materials and Methods

2.1. Materials

- *Pronase from Streptomyces griseus was obtained from Boehringer Manheim.*
- *Collagenase type II prepared from Clostridium Histolyticum was obtained from Worthington, Lakewood, NJ, US.*
- *Dulbecco's Modified Eagles Medium (DMEM) without sodium pyruvate with 450mg/ml glucose with pyridoxine was obtained from Invitrogen, Frankfurt, Germany.*
- *Gentamicin (1000x stock) was obtained from Invitrogen, Frankfurt, Germany.*
- *Foetal Calf Serum (FCS) was obtained from Invitrogen, Frankfurt, Germany.*
- *Phosphitan™C was obtained from Showa Denko, Tokyo, Japan.*
- *40µm Nylon Cell Strainers were obtained from BD Falcon, BD-Biosciences - Discovery Labware, Bedford, MA, US.*
- *Seaplaque Agarose was obtained from Fisher, Loughborough, UK.*
- *All general chemical reagents used to make up the dimethylmethylene blue (DMMB) solution was obtained from Sigma-Aldrich, Poole, Dorset, UK.*
- *Chondroitin sulphate C from shark cartilage was obtained from Sigma-Aldrich, Poole, Dorset, UK.*
- *Mutiwell plates were obtained from Elkay Laboratory Products UK Ltd., Basingstoke, UK.*
- *Labsystem Multiscan MS spectrophotometer was used.*
- *Lactate Assay Kit was obtained from Sigma-Aldrich, Poole, Dorset, UK. Beckman.*
- *Ultracentrifuge tubes were obtained from Beckman, London Road, Bucks, UK.*
- *Recombinant human ADAMTS-4 was a kind gift donated by Dr. Carl Flannery, Wyeth, Boston, US.*
- *Recombinant human MMP-13 was a kind gift from Dr. Peter Mitchell formerly at Pfiser, New York US, now at Eli Lilly, Indiana, US.*
- *Chondroitinase ABC was obtained from Sigma-Aldrich, Poole, Dorset, UK.*
- *Keratanase was obtained from Seikagaku / AMS Biotechnology, Abindon, UK.*
- *Keratanase II was obtained from Seikagaku / AMS Biotechnology, Abindon, UK.*

- *Gradient 4-12% Tris Glycine gels were obtained from Invitrogen, Frankfurt, Germany.*
- *Nitrocellulose membrane was obtained from Schleicher and Schuell Bioscience, Dassel Germany.*
- *Alkaline phosphatase linked goat anti-mouse secondary antibody was obtained from Promega, Madison, US.*
- *BC-3, BC-14, BC-13 and BC-4 monoclonal antibodies were all developed and characterised by members of this laboratory (Hughes et al., 1995, Hughes et al., 1992 and for reviews see Caterson et al., 1995 and Caterson et al., 2000).*
- *Heparin immobilised on 4% beaded agarose (activation epichlorohydrin) was obtained from Sigma-Aldrich, Poole, Dorset, UK.*
- *Imidodiacetic acid immobilised on cross-linked 4% beaded agarose (activation epoxy) were obtained from Sigma-Aldrich, Poole, Dorset, UK.*

2.2 Methods

2.2.1 Isolation of Porcine Articular Cartilage Chondrocytes

Porcine articular cartilage was obtained from 2-6 month old pig hock joints, which had been slaughtered at Ensors abattoir at Cinderford in the Forest of Dean. Hocks were obtained within 4-6 hours of slaughter. Chondrocytes were isolated using methods described by Hughes *et al.,* 1998. The hocks were firstly cleaned and skinned prior to dissection of the metacarpophalangeal joint and removal of cartilage tissue slices under asceptic conditions. The chondrocytes were isolated from their surrounding matrix firstly by digesting the cartilage slices in 0.1% (w/v) pronase in DMEM containing 50µg/ml gentamicin and 5% (v/v) Foetal Calf Serum (FCS) (7.5ml / g cartilage wet weight) for 3-4 hours at 37°C with constant agitation. Following removal of the pronase solution the tissue was further incubated at 37°C overnight with 0.04% (w/v) collagenase in DMEM containing 50µg/ml gentamicin and 5% (v/v) FCS (7.5ml / g cartilage wet weight) with constant agitation to free the cells from the collagen network.

The cells were filtered through a 40µm Nylon (Falcon) filter. The chondrocytes were pelleted by centrifugation at 1500rpm for 10 minutes, and then resuspended in 50ml of DMEM; this was repeated twice to wash the chondrocytes free of enzymes. The chondrocytes were resuspended in a known volume of DMEM a quantity of which was then taken and further diluted before being counted using a bright light haemocytometer.

The total cell number was calculated according to the equation:

$$\text{Number of cells} = \text{Average cell number} \times 10^4 \times \text{Total volume} \times \text{Dilution}$$

The chondrocytes were then resuspended at 12×10^6 cells / ml in DMEM with 50µg/ml gentamicin.

2.2.2 Preparation of Chondrocyte-Agarose Cultures

A 2% (w/v) solution of Seaplaque Agarose in DMEM containing 50µg/ml gentamicin was prepared and dissolved by heating before diluting to 1% (w/v) with DMEM containing 50µg/ml gentamicin. This 1% (w/v) agarose solution was plated at 2ml per 60mm plate and allowed to set at 4°C for 20-25 minutes before warming through at 37°C for 10-15 minutes to form a plug. Chondrocytes at 12 x 10^6 / ml were diluted 1:2 with 2% (w/v) agarose, resulting in a 1% (w/v) agarose solution containing 6 x 10^6 chondrocytes / ml, 1ml of this solution was overlaid onto the agarose plug and again allowed to set at 4°C for 10-15 minutes. Preculture medium of DMEM with 50µg/ml gentamicin, 10% (v/v) Foetal Calf Serum (FCS) and 25µg/ml Phosphitan™C (a stable form of ascorbate) was added at 4ml/plate and the cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, the medium was changed every 4-5 days. The chondrocytes were precultured for 14 or 21 days and then washed 3 x 20 minutes in serum free DMEM containing 50µg/ml gentamicin. The experimental conditions vary and are therefore described in the appropriate sections.

2.2.3 Extraction of Proteoglycans from Agarose Plugs

Matrix molecules, including proteoglycans, present in the agarose plugs were extracted by addition of 10ml guanidine extraction buffer (4M guanidine HCl, 50mM sodium acetate pH 5.8-6.8, 0.1M 6-amino-hexanoic acid, 5mM benzamidine HCl, 10mM ethylene diaminetetra acetic acid (EDTA) (tetrasodium salt), 1mM phenyl methyl sulphonyl fluoride (PMSF) to each 3ml plug for 48 hours at 4°C with constant agitation (as described in Roughley and White 1980). The mixture was dialysed exhaustively against MilliQ™ water before being spun at 15,000rpm for 30 minutes to remove the remaining agarose and the supernatant stored at -20°C until required. The remaining agarose plugs were subjected to alkaline β-elimination to extract any remaining GAGs by addition of 5ml 1M sodium hydroxide to each 3ml agarose plug and incubation at room temperature for 24 hours with constant agitation (adapted from Anderson *et al.,* 1964). This mixture was then dialysed exhaustively against MilliQ™ water. The samples were spun at 15,000rpm for 30 minutes to remove the remaining agarose and the supernatant stored at -20°C until required.

2.2.4 Analysis of Glycosaminoglycan Concentration using the Dimethylmethylene Blue Assay (DMMB)

Proteoglycan content of medium, guanidine extracts and β-eliminations from chondrocyte-agarose cultures was measured as sulphated GAG using the DMMB assay (Farndale *et al.,* 1986). In this assay, DMMB binds to the sulphate groups on glycosaminoglycans forming a dye-GAG complex. Formation of this complex produces a shift in the colour absorbance from blue to pink.

Standards ranging from 0-40µg/ml shark chondroitin sulphate C, and appropriately diluted unknown samples were added to a 96 well multiwell plate. 200µl of DMMB solution (32mg 1,9 DMMB, 20ml ethanol, 59ml 1M sodium hydroxide, 7ml 98% (v/v) formic acid and made up to 2L with MilliQ™ water) was added to the samples and the absorbance read immediately at 525nm on a Labsystem Multiscan MS Spectrophotometer. All standards were measured in triplicate and all samples were measured in duplicate.

2.2.5 Analysis of Lactate Concentration

Lactate assay was carried out using the Sigma lactate assay kit as a measure of metabolic activity and hence an indicator of cell viability during the preculture and experimental periods. The principal behind this kit is that lactic acid is converted to pyruvate and hydrogen peroxide by lactate oxidase. In the presence of hydrogen peroxide, peroxidase catalyses the oxidative condensation of chromogen precursors to produce a coloured dye with an absorption maximum of 540nm. The increase in absorbance is directly proportional to lactate concentration in the sample.

In a multiwell plate 5µl of lactate standards of 400, 300, 200, 100, 50 and 25µg/ml were placed in individual wells. 5µl of culture medium, diluted 1:10 with water, was also placed in individual wells. To each well was added 250µl of lactate reagent and incubated at room temperature for 5-10 minutes. The colour change was then read using the Labsystem multiscan MS spectrophotometer at 540nm. All standards were measured in triplicate and all samples were measured in duplicate.

2.2.6 Extraction and Purification of Aggrecan from Porcine Articular Cartilage

Articular cartilage was harvested from the metacarpophalangeal joints of 2-6 month old pigs as described in Section 2.2.1. Cartilage was finely diced prior to addition of guanidine extraction buffer (see Section 2.2.3) (10ml per gram cartilage wet weight) and incubation for 48 hours at 4°C with constant agitation. The extracted cartilage debris was removed, by centrifugation at 15,000rpm for 10 minutes, and discarded. The liquid supernatant was dialysed exhaustively against MilliQ™ water. The volume of liquid following dialysis was noted and a 1/10 volume of 10x sodium acetate buffer was added (500mM sodium acetate pH 7.5). Sufficient dry weights of the following proteinase inhibitors were added to give final concentrations of 0.1M 6-amino-hexanoic acid, 5mM benzamidine HCl, 10mM EDTA (tetrasodium salt) and 1mM PMSF. The density of the extract was adjusted to 1.5g/ml by addition of caesium chloride and aggrecan purified by ultracentrifugation in a Beckman L-60 Ultracentrifuge at 37,000rpm for 70 hours at 4°C. The extract was fractionated into 4 equal pools designated A1-A4, the lowest fraction A1 containing the purified aggrecan having a density >1.57g/ml and the highest A4 having a density of <1.4g/ml. The A1 fraction was adjusted to 4M guanidine HCl by the addition of sufficient solid guanidine HCl. The density was then adjusted to 1.5g/ml by the addition of caesium chloride solid and spun at 37,000rpm for 70 hours at 4°C in a Beckman L-60 Ultracentrifuge. The tubes were then fractionated into 4 equal portions designated A1D1-A1D4, the lowest fraction A1D1 having a density >1.57g/ml and the highest A1D4 having a density of <1.35g/ml. The A1D1 fraction was dialysed exhaustively against MilliQ™ water and analysed for sulphated GAG content by the DMMB assay as described in Section 2.2.4.

2.2.7 Digestion of A1D1 by Recombinant Human ADAMTS-4 and MMP-13 in Order to Generate Neoepitope Bearing Aggrecan Fragments

Sample of A1D1 (100μg GAG equivalents) were digested either with recombinant human ADAMTS-4 (1μg), to generate a positive control of aggrecan fragments bearing the amino- and carboxy-terminal neoepitopes ^{374}ARGSV... and ...NITEGE^{373}, respectively, or recombinant human MMP-13 (1μg activated by 100mM 4-aminophenylmercuric acetate [APMA]), in order to generate a positive control of aggrecan fragments bearing the amino- and carboxy-terminal neoepitopes ^{342}FFGV... and ...DIPEN^{341}, respectively, buffered by addition of a 1/10 volume of 10x digestion buffer (200mM tris HCl pH 7.5 containing 1M sodium chloride and 100mM calcium chloride) overnight at 37°C with constant agitation.

2.2.8 Western Blot Analysis of Aggrecan Fragments

Media, guanidine extracts and positive control samples equivalent to 40-100μg of sulphated GAG were adjusted to 0.1M tris acetate pH 6.5 by the addition of 1M tris acetate pH 6.5 prior to deglycosylation by addition of Chondroitinase ABC (0.001U per 10μg GAG), Keratanase (0.001U per 10μg GAG) and Keratanase II (0.00001U per 10μg GAG) and incubation at 37°C for 4-5 hours (as described in Hughes *et al.,* 1995). The samples were then dialysed exhaustively against MilliQ™ water and lyophilised on a speedvac. The samples were reconstituted, in Laemmli sample buffer (62.5mM tris HCl, pH 6.8 containing 4% (w/v) Sodium Dodecyl Sulphate (SDS), 20% (v/v) glycerol and 0.01% (w/v) bromophenol blue) (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed under reducing conditions on 4-12% Tris Glycine gels in running buffer (25mM trizma, 192mM glycine and 0.1% (w/v) SDS). The gels were then transferred onto Nitrocellulose membrane (0.22μ) in transfer buffer (25mM trizma pH 8.1-8.4 containing 192mM glycine and 20% (v/v) methanol) at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis. Membranes were blocked in 5% (w/v) Bovine Serum Albumen (BSA) in Tris Saline Azide (TSA - 50mM tris, 200mM sodium chloride pH 7.4 containing 0.02% (w/v) sodium azide) for a minimum of 1 hour at room temperature with rocking. The membranes were washed 3 x 10 minutes in TSA and incubated in the appropriate primary antibodies diluted 1:100 in 1% (w/v) BSA in TSA overnight at room temperature with rocking.

Media samples were probed with monoclonal antibodies BC-3 (which specifically recognises aggrecan metabolites bearing the amino-terminal aggrecanase generated interglobular domain neoepitope sequence ^{374}ARGSV...) and BC-14 (which specifically recognises aggrecan metabolites bearing the amino-terminal MMP-generated interglobular domain neoepitope sequence ^{342}FFGV...).

Metabolites from guanidine extracts of agarose plugs were probed with monoclonal antibodies BC-13 (which specifically recognises aggrecan metabolites bearing the carboxy-terminal aggrecanase generated interglobular domain neoepitope sequence ...NITEGE^{373}) and BC-4 (which specifically recognises aggrecan metabolites bearing the carboxy-terminal MMP-generated interglobular domain neoepitope sequence ...DIPEN^{341}).

The membranes were washed 3 x 10 minutes in TSA then incubated for 1 hour at room temperature with rocking in alkaline phosphatase conjugated goat anti-mouse secondary antibody diluted 1:7500 in 1% (w/v) BSA in TSA. The membranes were washed 3 x 10 minutes in TSA and developed in nitro blue tetrazolium (NBT - 50mg/ml in dimethylformamide) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP -- 50mg/ml in dimethylamide), 66μl NBT and 33μl BCIP per 10ml alkaline phosphatase (AP) buffer (100mM tris, 100mM sodium chloride, pH 9.55 containing 5mM magnesium chloride) (from Hughes *et al.,* 1998).

2.2.9 Detergent Extraction of Agarose Plugs

Each 3ml agarose plug was extracted in 4ml detergent extraction buffer (50mM Tris HCl, 100mM sodium chloride, pH 7 with 0.5% (v/v) nonidet P-40) for 24 hours at 4°C on a roller (as described by Gao *et al.,* 2002). The mixture was spun at 15,000rpm for 30 minutes at 4°C and the supernatant removed and stored at -80°C for later analysis. The remaining agarose was discarded.

2.2.10 Partial Purification of Media Samples from Chondrocyte-Agarose Cultures

- **Semi-Purification of Culture Medium using Heparin Affinity Binding**

Aliquots (100µl) of heparin-sepharose gel suspension were placed in individual eppendorfs and washed 5 x 500µl with 100mM tris HCl pH 7.5 containing 50mM sodium chloride and 0.05% (v/v) brij (here named equlibriation buffer). Each bead aliquot was incubated with a 500µl aliquot of culture medium, harvested from the agarose culture experiments, on a mixer at 4°C for 30-35 minutes. Following this the supernatant was removed and stored on ice prior to further purification (see below). The beads were washed, to remove non-bound material, 5 x 100µl with equlibriation buffer and the first wash was combined with the column supernatant and stored on ice prior to further purification (see below). The beads were then eluted in 100µl of 0.8M sodium chloride, 100mM tris HCl, pH 7.5 with 0.05% (v/v) brij (here named elution buffer). Samples were stored at -80°C for later analysis.

- **Semi-Purification of Heparin-Sepharose Non-bound Fractions Using Zinc Chelation**

Aliquots (200µl) of imidodiacetic acid gel suspension were placed in individual eppendorfs and washed 5 x 200µl with MilliQ™ water before chelation using 2 x 100µl of 1mg/ml zinc chloride for 5 minutes each time. The beads were washed 2 x 50µl in 0.5M sodium chloride, 20mM tris HCl pH 7.5 (buffer 1) then 2 x 500µl in 0.5M sodium chloride, 20mM tris HCl pH 7.5 with 5mM calcium chloride (start buffer). The beads were incubated with 500µl of the supernatant from the Heparin-Sepharose column combined with 100µl of the first column wash from the heparin column and 60µl of 5M sodium chloride, 200mM tris HCl pH7.5 with 50mM calcium chloride for 5-10 minutes on ice. The column supernatant was removed and stored at -80°C, the beads were then washed 5 x 100µl in start buffer before eluting with 5 x 100µl of 0.5M sodium chloride, 20mM tris HCl pH 7.5 containing 1mM calcium chloride and 35mM imidazole. All eluents were stored at -80°C until required.

Chapter 3: Composition of Extracellular Matrix Secreted by Chondrocyte-Agarose Cultures

3.1 Introduction

In order to investigate the sequestration and activity of ADAMTS-4 and -5 a model system of chondrocytes embedded in agarose was utilised. This system involves preculture of chondrocytes in agarose with foetal calf serum and Phosphitan™C (a stable ascorbate analogue) to allow secretion of an extracellular matrix. In this chapter partial characterisation of this newly synthesized matrix is described using previously characterised monoclonal antibodies (M'Abs) which recognise carbohydrate moieties present on proteoglycans of the articular cartilage extracellular matrix (see Figure 3.1).

Chondroitin-4-sulphate 'stubs' are recognised on deglycosylated aggrecan, decorin and biglycan by M'Ab 2B6 (Caterson *et al.,* 1985). Chondroitin-6-sulphate 'stubs' are recognised on deglycosylated aggrecan by M'Ab 3B3+ (Caterson *et al.,* 1995). The (+) indicates deglycosylation with Chondroitinase ABC. A mimotope (a biochemical structure that mimics the epitope recognised by a given antibody) (Geysen *et al.,* 1988), containing a saturated glucuronic acid residue at the non-reducing terminal (Caterson *et al.,* 1990), that occurs in chondroitin sulphate chains of proteoglycans isolated from osteoarthritic cartilage is recognised in non-deglycosylated aggrecan by the M'Ab 3B3(-) (Visco *et al.,* 1993). The (-) indicates that no predigestion has been carried out. Proteoglycans containing the 3B3(-) mimotope at the non-reducing terminal of chondroitin sulphate glycosaminoglycans occur at low frequency in proteoglycans isolated from normal cartilage. However, its expression is much more prevalent in proteoglycans isolated from osteoarthritic cartilage (Carney *et al.,* 1992, Slater *et al.,* 1995, and Caterson *et al.,* 1991). Keratan sulphate chains on aggrecan, fibromodulin and lumican are recognised by M'Ab 5D4 (Caterson *et al.,* 1983). Thus the similarities and differences between the matrix synthesised by chondrocytes in agarose and a native articular cartilage extracellular matrix may be determined.

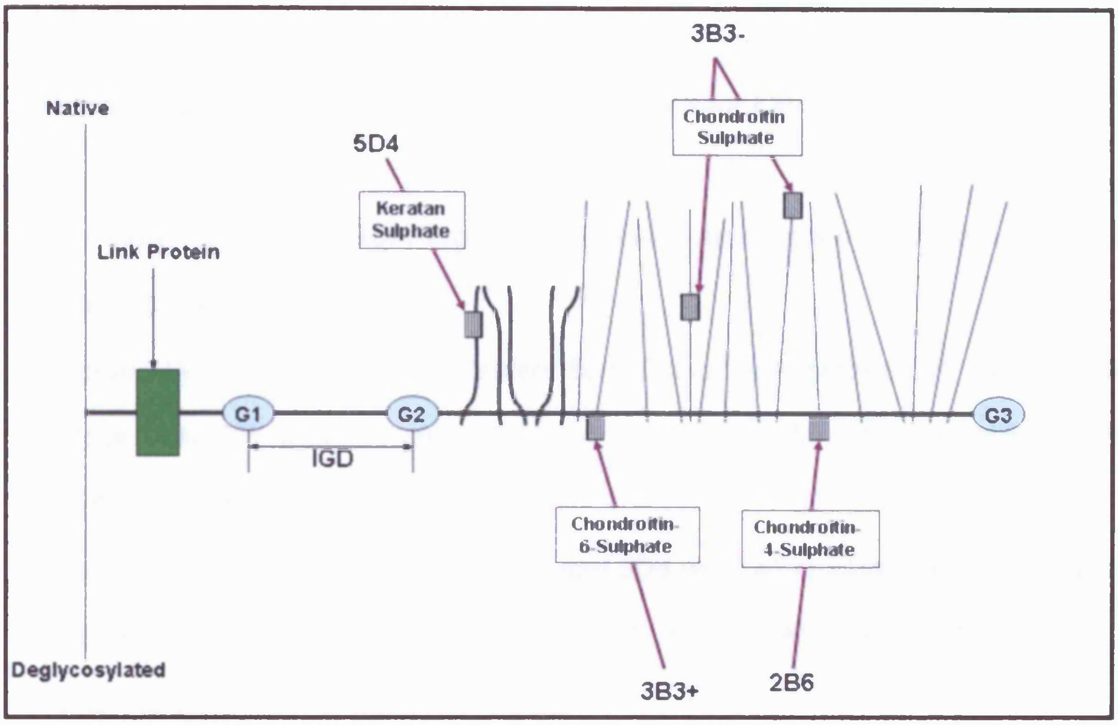

Figure 3.1 Diagrammatic representation of the structure of aggrecan showing its' glycosaminoglycan chains and the specific monoclonal antibodies which recognise epitopes within the chains and on the 'stubs' resulting from digestion of the glycosaminoglycan chains with Chondroitinase ABC.

3.2 Materials

- *Horse serum was obtained from Vector Laboratories Inc, Burlingame, CA, US.*
- *Horse anti-mouse antibody-horse radish peroxidase conjugate was obtained from Vector Laboratories Inc, Burlingame, CA, US.*
- *Peroxidase substrate solution was obtained from Vector Laboratories Inc, Burlingame, CA, US.*
- *Avidin Biotin Complex (ABC)-Peroxidase elite kit was obtained from Vector Laboratories Inc, Burlingame, CA, US.*
- *Phosphate Buffered Saline (PBS) tablets from Oxoid, Basingstoke, UK, were dissolved 1 per 100ml MilliQ™ water to give 10mM phosphate, 2.7mM potassium chloride, 137mM sodium chloride pH 7.4.*
- *Monoclonal Antibodies (M'Abs) 2B6, 3B3 and 5D4 were all produced and characterised by members of this laboratory (Caterson et al., 1983, 1985, 1990, 1991 and 1995).*
- *All other reagents were of analytical grade.*

3.3 Methods

3.3.1 Preparation of Chondrocyte-Agarose Cultures

Chondrocyte agarose cultures were prepared and precultured for 14 days as described in Chapter 2 Section 2.2.1 and 2.2.2. Following this preculture period the plates were washed 3 x 20 minutes in serum free DMEM with 50µg/ml gentamicin before culture in serum free DMEM with 50µg/ml gentamicin and 25µg/ml Phosphitan™C for 96 hours.

Following the 96 hour culture period the medium was removed and stored frozen. The agarose was sliced and wrapped in foil before being snap frozen in liquid nitrogen and stored at -20°C.

3.3.2 Histological Analysis

Cryosections of the agarose pieces (30µm in thickness) were cut using an automated Bright Cryostat and mounted on amino propyl triethoxysilane (APES) coated slides. These slides were prefixed in -20°C methanol then air dried face up at room temperature for 2-3 hours to facilitate adhesion. The slides were then wrapped in foil and stored at -20°C until required.

Slides were equilibrated to room temperature and sections were fixed in freshly prepared 4% (w/v) paraformaldehyde in Phosphate Buffered Saline (PBS) for 10 minutes at room temperature before being washed (3 x 10 minutes) in PBS containing 0.01% tween-20 (PBS-T). The sections were flooded with the appropriate buffer (see Table 3.1) and allowed to equilibrate for 10 minutes. The sections were incubated at 37°C for 2 hours in the necessary enzyme(s); for 2B6 and 3B3+ Chondroitinase ABC, Keratanase and Keratanase II digestion, for 5D4 Chondroitinase ABC digestion only and for 3B3- no digestion (Chondroitinase 0.5 Units/ml, Keratanase 0.5 Units/ml and Keratanase II 0.005 Units/ml) (see Table 3.1).

The sections were washed (3 x 5 minutes) in PBS-T and any non-specific binding was blocked by incubating sections in 5% (v/v) normal horse serum in PBS-T for 20 minutes at room temperature. Primary M'Abs 2B6, 3B3 and 5D4 were diluted in 2% (v/v) horse serum in PBS-T to 1:1600, 1:1000 and 1:1500, respectively (see Table 3.1). M'Abs were incubated on the slides for 1 hour at room temperature. Following 3 x 10 minute washes in PBS-T sections were incubated with biotinylated anti-mouse secondary antibody diluted 1:200 in 1% (v/v) horse serum in PBS-T for 1 hour at room temperature. The sections were washed (3 x 10 minutes) before incubation in Vector's Avidin Biotin Complex (ABC) reagent for 1 hour at room temperature. Sections were washed (3 x 10 minutes) in PBS-T before applying Vector's 3, 3'-Diaminobenzidine (DAB) substrate solution for 5 minutes to develop the coloured product. The reaction was stopped by washing the sections in water before counterstaining to show the nuclei with Mayer's Haematoxylin. Sections were again washed in water then dehydrated through alcohols, cleared in xylene and mounted under coverslips in p-xylene-bis (N-pyridinium bromide) (DPX mountant).

Table 3.1 Monoclonal antibodies used for immunohistochemistry, their pre-treatments, dilutions and the epitope they recognise.

MONOCLONAL ANTIBODY	PRE-DIGECTION ENZYMES & BUFFERS	MONOCLONAL ANTIBODY DILUTION	EPITOPE RECOGNISED
3B3(-)	None	1:1000 1 hour RT	Native chondroitin sulphate
3B3(+)	Chondroitinase ABC, Keratanase and Keratanase II in 100mM tris acetate pH 6.5 with 5mM 1,10 phenanthroline	1:1000 1 hour RT	chondroitin-6-sulphate 'stubs'
2B6		1:1600 1 hour RT	chondroitin-4-sulphate 'stubs'
5D4	Chondroitinase ABC in 100mM tris acetate pH7.8 with 5mM 1,10 phenanthroline	1:1500 1hour RT	keratan sulphate

3.4 Results

Cryosections of chondrocyte-agarose cultures precultured with Foetal Calf Serum (FCS) and Phosphitan™C for 14 days to establish an extracellular matrix were immunostained with a variety of GAG recognising M'Abs to assess the composition and morphology of the proteoglycans secreted by the chondrocytes. The antibodies used and the epitopes they recognise on the GAG chains are listed in Table 3.2.

All four of the monoclonal antibodies utilised resulted in strong immunopositive staining pericellularly with paler more diffuse staining within the interterritorial matrix. This indicates a dense proteoglycan containing extracellular matrix to have been secreted by the chondrocytes suspended in agarose during the preculture period. For all four M'Abs control sections were stained in the absence of primary antibody or with preimmunised mouse serum and in all four cases these controls were blank indicating no detectable non-specific binding (Figures 3.2 – 3.5 A and B). The immunopositive staining seen with M'Abs 2B6 and 5D4 (Figures 3.2C and 3.5C, respectively) indicate that the secreted matrix contains proteoglycan, since 2B6 recognises chondroitin-4-sulphate 'stubs' present on deglycosylated proteoglycans such as aggrecan, decorin and biglycan (Caterson *et al.,* 1985) and 5D4 recognises epitopes within the keratan sulphate chains of aggrecan, fibromodulin and lumican (Caterson *et al.,* 1983). The dark immunopositive staining seen with M'Abs 2B6 and 3B3+ (Figures 3.2C and 3.3C, respectively) indicates that the majority of the proteoglycan present is in the form of aggrecan since 2B6 recognises chondroitin-4-sulphate 'stubs', and 3B3+ recognises chondroitin-6-sulphate 'stubs', present on deglycosylated aggrecan (Caterson *et al.,* 1985, and 1995, respectively). Interestingly, immunopositive staining was detected for 3B3- (Figure 3.4C). The presence of 3B3- immunopositive staining in these sections indicates epitopes to be present in the chondroitin sulphate chains of aggrecan which are characteristic of newly synthesised aggrecan or cartilage undergoing tissue remodelling (Visco *et al.,* 1993, Carney *et al.,* 1992, Slater *et al.,* 1995, and Caterson *et al.,* 1991).

Table 3.2 Monoclonal antibodies used in the immunohistochemical analysis of the proteoglycans present in agarose plugs from the chondrocyte-agarose cultures and the epitopes they recognise within the GAG chains and on the 'stubs' resulting from digestion of the glycosaminoglycan chains with Chondroitinase ABC.

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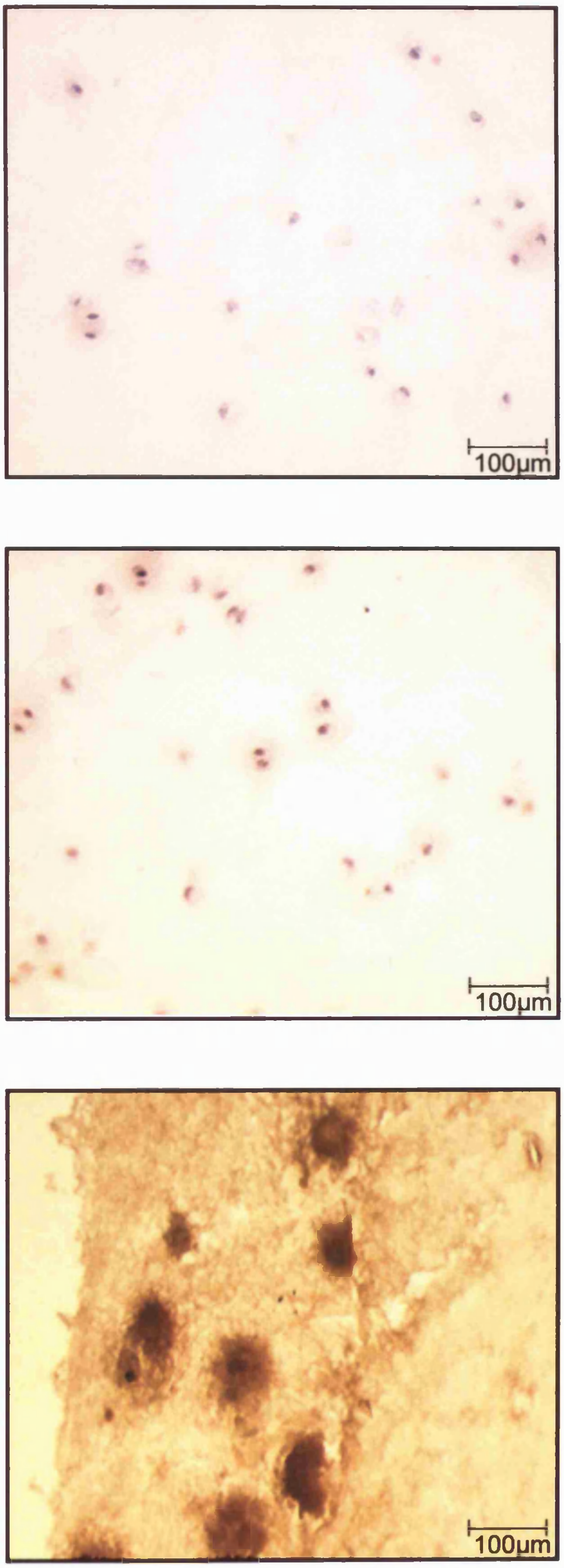
MONOCLONAL ANTIBODY	**EPITOPE RECOGNISED**
2B6	Chondroitin-4-sulphate 'stubs' on aggrecan, decorin & biglycan
3B3(-)	Chondroitin-sulphate characteristic of newly synthesised aggrecan
3B3(+)	Chondroitin-6-sulphate 'stubs' on aggrecan
5D4	Keratan sulphate on aggrecan, fibromodulin & lumican

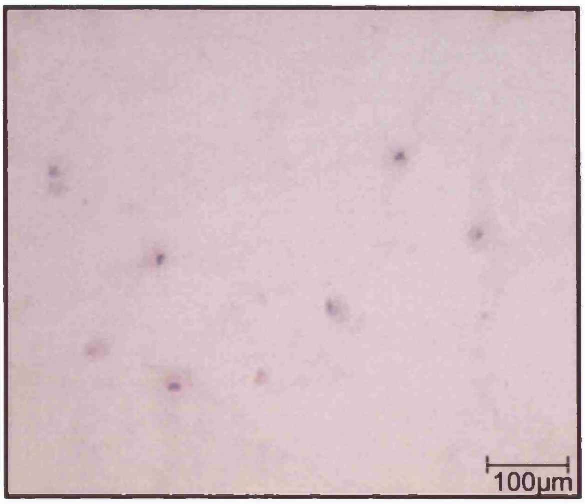


(A) No primary antibody control

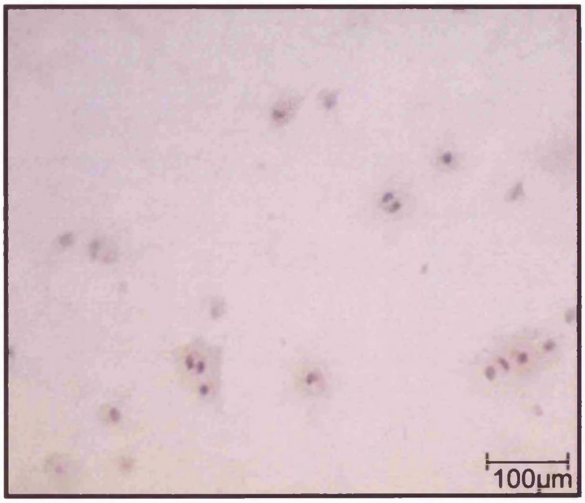
(B) Preimmunised mouse serum antibody control

(C) 2B6 M'Ab

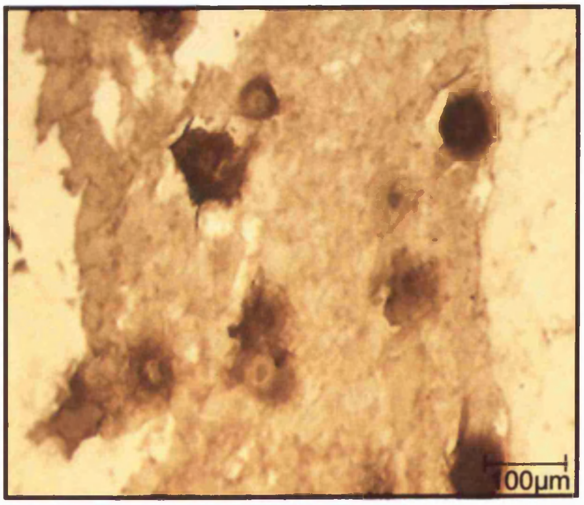
Figure 3.2 Immunohistochemical analysis of chondroitin-4-sulphate stubs present in deglycosylated cryosections of chondrocyte-agarose cultures (A) No primary antibody control section, (B) Preimmunised mouse serum antibody control section and (C) 2B6 stained section



(A) No primary antibody control

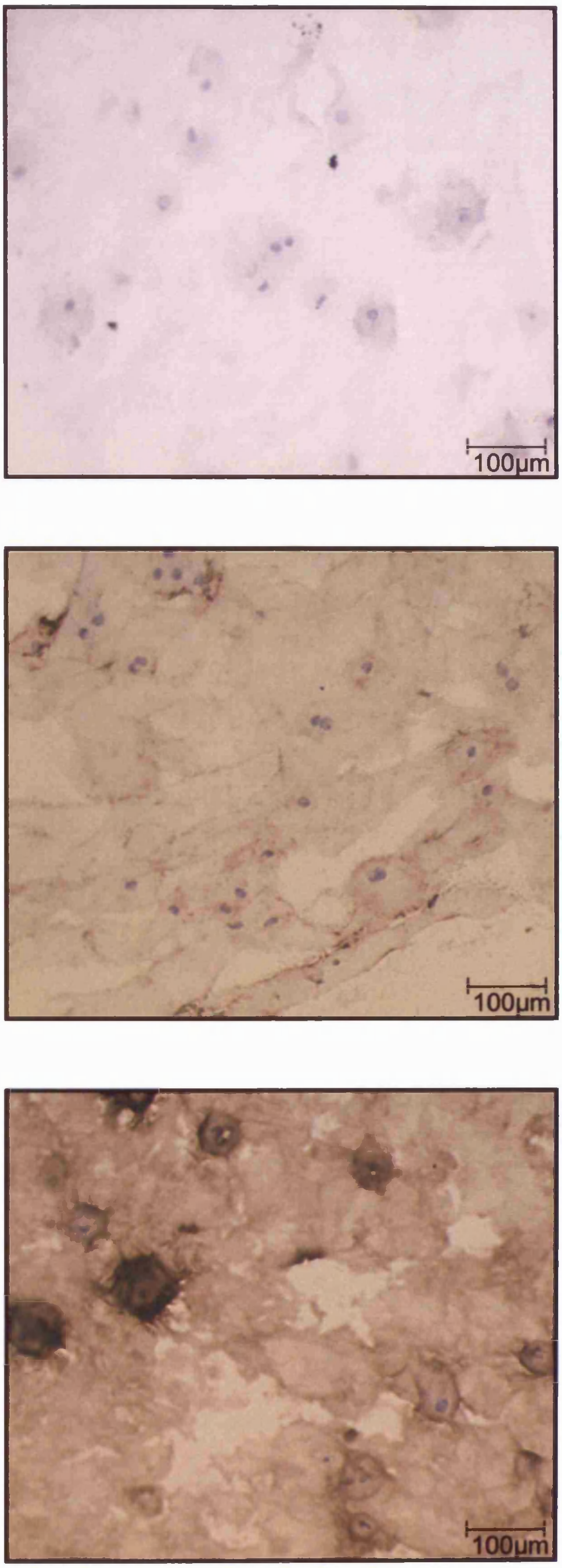


(B) Preimmunised mouse serum antibody control



(C) 3B3+ M'Ab

Figure 3.3 Immunohistochemical analysis of chondroitin-6-sulphate stubs present in deglycosylated cryosections of chondrocyte-agarose cultures (A) No primary antibody control section, (B) Preimmunised mouse serum antibody control section and (C) 3B3+ stained section

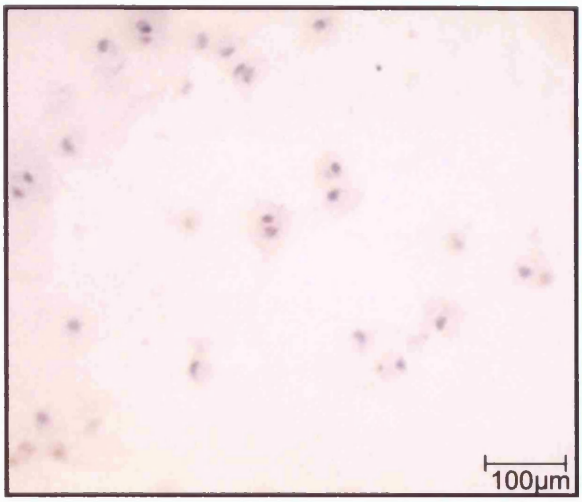


(A) No primary antibody control

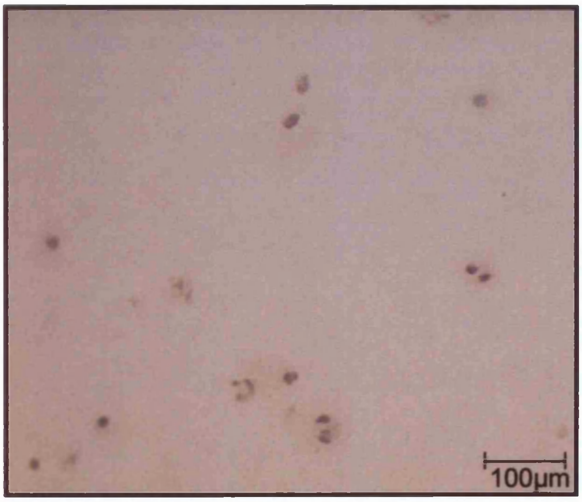
(B) Preimmunised mouse serum antibody control

(C) 3B3- M'Ab

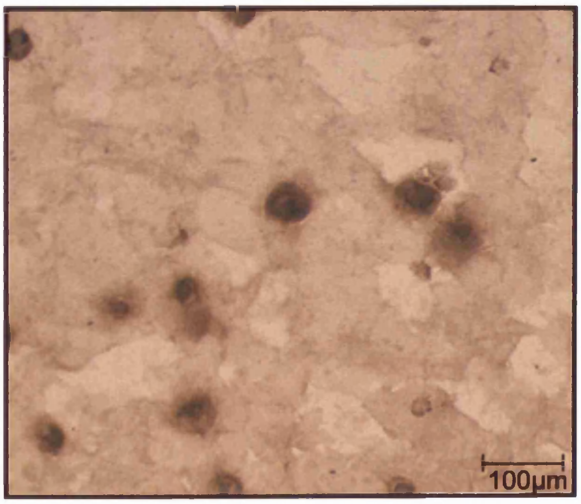
Figure 3.4 Immunohistochemical analysis of chondroitin sulphate stubs on newly synthesised aggrecan present in cryosections of chondrocyte-agarose cultures (A) No primary antibody control section, (B) Preimmunised mouse serum antibody control section and (C) 3B3- stained section



(A) No primary antibody control



(B) Preimmunised mouse serum antibody control



(C) 5D4 M'Ab

Figure 3.5 Immunohistochemical analysis of keratan sulphate chains present in cryosections of Chondroitinase ABC digested chondrocyte-agarose cultures (A) No primary antibody control section, (B) Preimmunised mouse serum antibody control section and (C) 5D4 stained section

3.5 Discussion

Staining of agarose sections with various M'Abs to epitopes within glycosaminoglycan chains and on the 'stubs' resulting from digestion of the GAG chains with Chondroitinase ABC indicate the establishment of an extracellular matrix containing proteoglycans, including aggrecan, to have been secreted around the chondrocytes embedded in agarose (Figures 3.2C, 3.3C and 3.5C). This matrix is most dense in the pericellular region with a sparser matrix penetrating further out into the agarose. This is similar to the matrix described by Ayedelotte and Kuettner in the culture system of bovine chondrocytes embedded in agarose (Ayedelotte and Kuettner 1988). Some of the aggrecan monomers present contain epitopes characteristic of newly synthesised aggrecan recognised by M'Ab 3B3- (Figure 3.4C). This pre-established extracellular matrix will be utilized as a model system to investigate the enzymes involved in the catabolism of aggrecan especially ADAMTS-4 and -5.

3.6 Summary

- A matrix rich in aggrecan was secreted by chondrocytes embedded in agarose during the preculture period in the presence of serum

Chapter 4: Investigation of the Effects of IL-1α on the Aggrecan Present in the Extracellular Matrix Secreted by Chondrocyte-Agarose Cultures

4.1 Introduction

The extracellular matrix of chondrocyte-agarose cultures described in Chapter 3 will be utilized as a model system to investigate the enzymes involved in extracellular matrix catabolism of aggrecan. In order to determine the effect of cytokines on this pre-established matrix, the chondrocyte-agarose cultures were treated with interleukin-1α (IL-1α) following 21 days of preculture (an adaptation of the investigation carried out by Aydelotte and Kuettner 1988). In cartilage IL-1α retards synthesis and secretion of matrix macromolecules and can induce matrix proteases such as ADAMTS-4 and -5 (Arner *et al.,* 1999) and MMPs (Cawston *et al.,* 1999). IL-1α signalling is extremely rapid and within a few minutes of binding to the cell IL-1α can induce several biochemical processes (Dinarello 1996). High levels of IL-1 in human joint effusions have been proposed to be responsible for the cartilage degeneration seen in inflammatory joint disease (Tortorella *et al.,* 1999). IL-1α was used as the catabolic agent as it is known to mimic the degradative process involved in the catabolism of articular cartilage in diseases such as osteoarthritis and rheumatoid arthritis. The effect of this cytokine treatment on the chondrocyte-agarose matrix will be analysed using the DMMB assay to determine the proportion of the sulphated GAG in the matrix released to the medium following cytokine treatment. The composition of this released sulphated GAG will be analysed by Western blotting using M'Abs which recognise neoepitopes generated by cleavage within the interglobular domain (IGD) of the core protein of aggrecan (see Figure 4.1).

During aggrecan catabolism cleavage sites are utilised within the IGD of the core protein. The Asn^{341} - Phe^{342} bond is cleaved by a number of members of the MMP family (Fosang *et al.,* 1991, 1992, 1993, 1994, 1996 and 1998, Lark *et al.,* 1995, Stracke *et al.,* 2000, and Little *et al.,* 1999) and results in the amino- and carboxy-terminal neoepitopes ^{342}FFGV... and ...DIPEN^{341}, respectively. The ^{342}FFGV... neoepitope is recognised by M'Ab BC-14 and the ...DIPEN^{341} neoepitope is recognised by M'Ab BC-4 (see Figure 4.1). The Glu^{373} -Ala^{374} bond can be cleaved by ADAMTS-4 and -5 (see Chapter 1 Section 1.5.2) and results in the amino- and carboxy-terminal neoepitopes ^{374}ARGSV... and ...NITEGE^{373}, respectively. The ^{374}ARGSV... neoepitope is recognised by M'Ab BC-3 and the ...NITEGE^{373} neoepitope is recognised by M'Ab BC-13 (see Figure 4.1). Thus the effect of IL-1α on the rate of proteoglycan release from the matrix may be determined, using the DMMB assay, as well as which enzymes are responsible for matrix catabolism in this culture system by Western blot analysis with specific neoepitope antibodies (Figure 4.1).

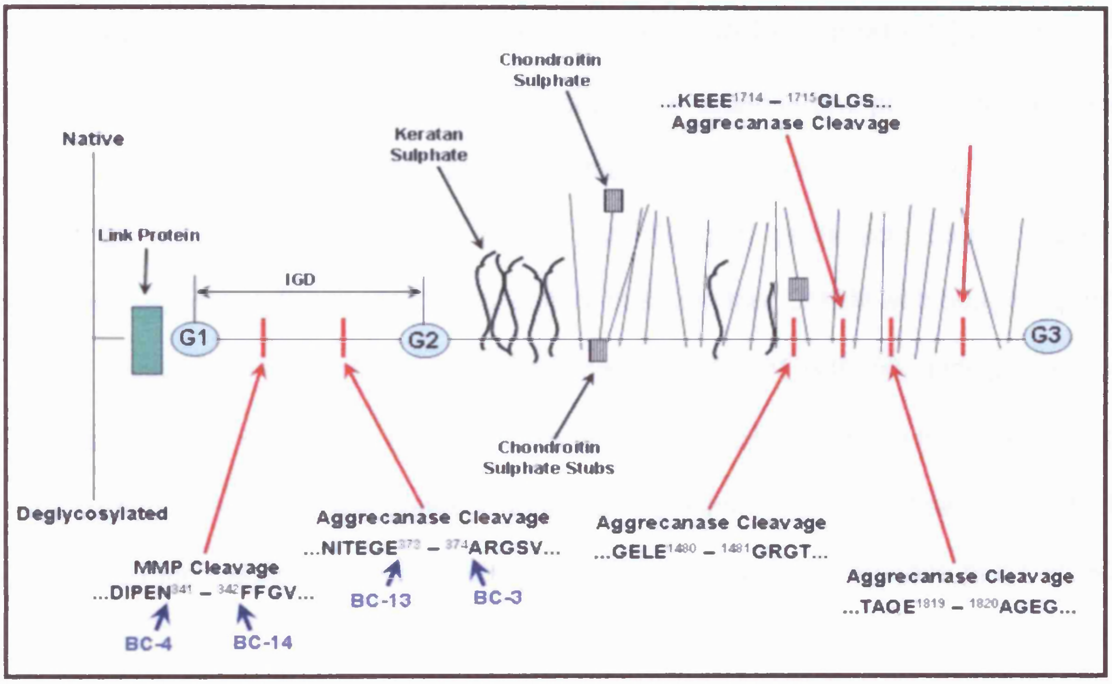


Figure 4.1 Diagram of aggrecan structure showing GAG chains, cleavage sites, neoepitopes and specific monoclonal antibodies (M'Abs)

4.2 Materials

- *Interleukin-1 (IL-1)α human recombinant was obtained from Tebu-Bio Ltd., Peterborough, UK.*
- *All other reagents are listed in Chapter 2 Section 2.1.*

4.3 Methods

4.3.1 Treatment of Chondrocyte-Agarose Cultures with Interleukin-1 (IL-1)α

Porcine articular chondrocytes were isolated, embedded in agarose and precultured as described in Chapter 2 Section 2.2.1 and 2.2.2. Following the 21 day preculture period the plates were washed 3 x 20 minutes in serum free DMEM with 50µg/ml gentamicin before experimental conditions were set up as follows: (A) Control culture in serum free DMEM with 50µg/ml gentamicin and 25µg/ml Phosphitan™C or (B) Serum free medium (DMEM with 50µg/ml gentamicin and 25µg/ml Phosphitan™C) containing 10ng/ml IL-1α, for 24 to 120 hours. Each treatment and culture period was carried out on triplicate plates in triplicate experiments giving a total n of 9. Following the culture period the medium was removed and stored along with the agarose plugs at -80°C for further analysis.

4.3.2 Analysis of Experimental Medium Collected At Time Intervals Following Treatment in Serum Free Medium

The metabolic activity of the chondrocytes during the experimental period was analysed using the Lactate assay as described in Chapter 2 Section 2.2.5. Proteoglycans present in the agarose plugs were extracted as described in Chapter 2 Section 2.2.3. The concentration of sulphated GAG released to the culture medium, and present in the guanidine extracts and alkaline β-eliminations was analysed using the DMMB assay described in Chapter 2 Section 2.2.4.

Western blot analysis was carried out on media and guanidine extracts as described in Chapter 2 Section 2.2.8. Positive controls for these analyses were prepared as described in Chapter 2 Sections 2.2.6 and 2.2.7.

4.4 Results

4.4.1 Analysis of Lactate and Sulphated GAG Released to Culture Medium at Time Intervals Following Treatment in Serum Free Medium

Media samples were analysed using the Lactate assay kit. The results of this analysis show treatment with IL-1α to have no effect on the metabolic activity of the chondrocytes present in the culture system (results not shown).

The amount of sulphated GAG released to the medium during the treatment time was determined using the Dimethyl Methylene Blue (DMMB) assay. The agarose plugs were extracted with 4M guanidine HCl at the end of each treatment time, in order to release intact proteoglycan, followed by alkaline β-elimination to release any remaining GAG chains (the alkaline β-elimination causes release of the GAG chains from the proteoglycan core protein and subsequent removal from the agarose matrix). GAG content was determined in both the guanidine extracted and β-eliminated agarose plug samples. The total sulphated GAG per culture was then calculated as the sum of the GAG concentrations measured in the medium, guanidine extracted and β-eliminated plug samples. The raw data results of all of these analyses are shown in Table 4.1. In addition the percentage of the total sulphated GAG present released into the media was calculated as μg GAG in medium / total GAG per culture plate x 100. The total GAG per plate was decreased in cultures treated with IL-1α compared to control cultures (Table 4.1). An Anderson-Darling test for normality showed the differences between the total GAG present in control and IL-1α treated cultures to be normally distributed (p-value 0.782). Comparison of the total GAG present per plate in control and IL-1α treated cultures using a paired t-test gave a p-value of 0.001. Therefore, the increased concentration of GAG per plate detected in control cultures compared to those treated with IL-1α was statistically significant. This may be due to known ability of IL-1α to decrease proteoglycan synthesis.

Table 4.1 Tabulated results of three separate experiments showing the mean GAG (μg) released into the culture medium and from guanidine extracted and alkaline β-eliminated agarose plugs of triplicate control and IL-1α (10ng/ml) treated cultures collected over a 120 hour time period. From these mean results the total GAG (μg) per plate was calculated and thus the percentage of the total GAG released to the culture medium during each treatment and treatment time (%).

	Treatment and Time	GAG (μg/plate)				Percentage of total GAG Released to medium (%)
		Media	Guanidine Extract	Alkaline β-Elimination	Total	
Experiment 1	Control 24 hrs	46.78	28.52	114.60	189.9	**24.6**
	IL-1 24 hrs	227.92	1.64	54.79	284.4	**80.1**
	Control 48 hrs	124.04	171.30	150.91	446.3	**27.8**
	IL-1 48 hrs	256.16	11.33	66.14	333.6	**76.8**
	Control 72 hrs	145.72	199.02	110.80	455.5	**31.9**
	IL-1 72 hrs	242.10	0.00	18.61	260.7	**92.9**
	Control 96 hrs	148.76	207.15	79.70	435.6	**34.2**
	IL-1 96 hrs	345.10	0.00	9.35	354.5	**97.3**
	Control 120 hrs	268.26	234.89	37.40	540.6	**49.6**
	IL-1 120 hrs	463.4	0.00	4.4	467.8	**99.1**
Experiment 2	Control 24 hrs	43.95	90.9	266.1	400.95	**10.96**
	IL-1 24 hrs	196.70	2.4	84.2	283.3	**69.43**
	Control 48 hrs	47.15	98.4	268.8	414.35	**11.38**
	IL-1 48 hrs	278.75	10.1	69.95	358.8	**77.68**
	Control 72 hrs	77.45	145.1	276.9	499.45	**15.51**
	IL-1 72 hrs	451.90	7.4	96.5	555.8	**81.31**
	Control 96 hrs	113.10	229.5	320.5	663.1	**17.06**
	IL-1 96 hrs	570.60	0.0	61.15	631.75	**90.32**
	Control 120 hrs	126.45	371.1	296.7	794.25	**15.92**
	IL-1 120 hrs	490.40	0.0	57.9	548.3	**89.44**
Experiment 3	Control 24 hrs	48.90	90.4	178.23	317.53	**15.4**
	IL-1 24 hrs	155.28	5.6	45.61	206.49	**75.2**
	Control 48 hrs	85.00	100.2	237.69	422.89	**20.1**
	IL-1 48 hrs	228.32	11.3	55.37	294.99	**77.4**
	Control 72 hrs	121.70	156.3	224.89	502.89	**24.2**
	IL-1 72 hrs	256.16	7.1	53.38	316.64	**80.9**
	Control 96 hrs	129.38	230.6	130.10	490.08	**26.4**
	IL-1 96 hrs	253.24	4.2	39.79	297.23	**85.2**
	Control 120 hrs	191.86	356.2	89.35	637.41	**30.1**
	IL-1 120 hrs	288.60	0.0	26.47	315.07	**91.6**

The yield of intact proteoglycan extracted from the agarose plugs by guanidine extraction was relatively low in comparison with the amount of isolated GAG chains extracted by β-elimination (see Table 4.1). This may indicate a tight association between aggrecan present in the agarose plugs and other matrix molecules with treatment of agarose plugs by β-elimination being sufficient to release the GAG chains from the matrix.

An Anderson-Darling test for normality showed the differences between the GAG released to the medium in control and IL-1α treated cultures to be normally distributed (p-value 0.553). A paired t-test comparing the amount of sulphated GAG released to the medium of control and IL-1 treated cultures gave a p-value of <0.00001showing the increased GAG release seen in the IL-1 treated cultures compared to controls to be statistically significant (see Figure 4.2).

An Anderson-Darling test for normality gave p-values of 0.362 and 0.511 for GAG released to the medium of control and IL-1 treated cultures, respectively, indicating the data to be normally distributed. A p-value of 0.493 was obtained from a one way Analysis of Variance (ANOVA) for comparison of the GAG released to the medium of control cultures at the different treatment time points. This indicates no statistical difference in the GAG released to the medium of control cultures between the treatment times. A p-value of 0.005 was obtained from a one way ANOVA for comparison of the GAG released to the medium of IL-1α treated cultures at the treatment times tested. This indicates a statistically significant difference in the percentage of the total GAG released to the medium at the different treatment points in IL-1α treated cultures. Since treatment time in the presence of IL-1α significantly affects the proportion of the total sulphated GAG released to the medium, treatment time must be considered an important factor when analysing GAG release in IL-1α treated chondrocyte-agarose cultures.

a Individual 95% Confidence Intervals for Percentage of Total GAG Released to Medium in Control vs IL-1α (10ng/ml) Treated Cultures:

Treatment	Mean Percentage of Total GAG Released in Treatment Time (%)
Control	23.7
IL-1α	84.3

```
         -+---------+---------+---------+---------+
Control   (-*-)
IL-1α                                   (-*-)
         -+---------+---------+---------+---------+
          20        40        60        80        100
```

Mean and Spread of Total GAG Released in Treatment Time (%)

b Individual 95% Confidence Intervals for Percentage of Total GAG Released to Medium in Control Cultures at Different Treatment Times:

Treatment Time (hours)	Mean Percentage GAG Released in Control Cultures in Treatment Time (%)
24	16.99
48	19.76
72	23.87
96	25.89
120	31.87

```
      --------+---------+---------+--------
24hr (----------*----------)
48hr    (----------*----------)
72hr       (----------*----------)
96hr        (----------*----------)
120hr            (----------*----------)
      --------+---------+---------+--------
              12        24        36
```

Mean and Spread of Total GAG Released in Control Cultures in Treatment Time (%)

c Individual 95% Confidence Intervals for Percentage of Total GAG Released to Medium in IL-1α (10ng/ml) Treated Cultures in Different Treatment Times:

Treatment Time (hours)	Mean Percentage GAG Released in IL-1α Treated Cultures in Treatment Time (%)
24	74.91
48	77.29
72	85.04
96	90.94
120	93.38

```
      --+---------+---------+---------+----
24hr (------*------)
48hr    (-----*------)
72hr           (------*------)
96hr                 (------*------)
120hr                  (-----*------)
      --+---------+---------+---------+----
        70        80        90        100
```

Mean and Spread of Percentage of Total GAG Released to Medium in IL-1α Treated Cultures in Treatment Time (%)

Figure 4.2 (a) Shows the mean, and the spread either side of the mean, percentage of the total GAG per plate released to the medium in cultures treated in the absence (control) and presence of IL-1α (10ng/ml) at all time points (b) Shows the mean and the spread either side of the mean, percentage of the total GAG per plate released to the medium in control cultures at the different time points tested, and (c) Shows the mean, and the spread either side of the mean, percentage of the total GAG per plate released to the medium in IL-1α (10ng/ml) treated cultures at the different time points tested.

The mean percentage of the total GAG per culture plate released to the medium during the treatment time was expressed as a histogram and is shown in Figure 4.3.

In control cultures only 20 - 30% of the total sulphated GAG present in each plate was released into the medium following 120 hours of treatment (see Figure 4.3, and Table 4.1 for raw data). In contrast, the release of sulphated GAG to the medium in the cultures exposed to IL-1α occurred rapidly with over 70% of the total GAG being lost to the medium in the first 24 hours. This percentage release increased further to 80 - 90% of the total sulphated GAG present being released into the medium by 120 hours of treatment (Figure 4.3 and Table 4.1 for raw data).

Sulphated GAG concentration, measured by the DMMB assay, is routinely taken to reflect GAG chains on aggrecan; yet, other matrix proteoglycans containing GAG chains such as biglycan and decorin may also be present in the samples analysed and contribute to GAG measured using the assay. However, the majority of the proteoglycan present in the matrix secreted by chondrocytes embedded in agarose was shown to be composed of aggrecan in Chapter 3, the contribution of sulphated GAG from other proteoglycans is assumed to contribute minimally to the concentrations of GAG measured using the DMMB assay.

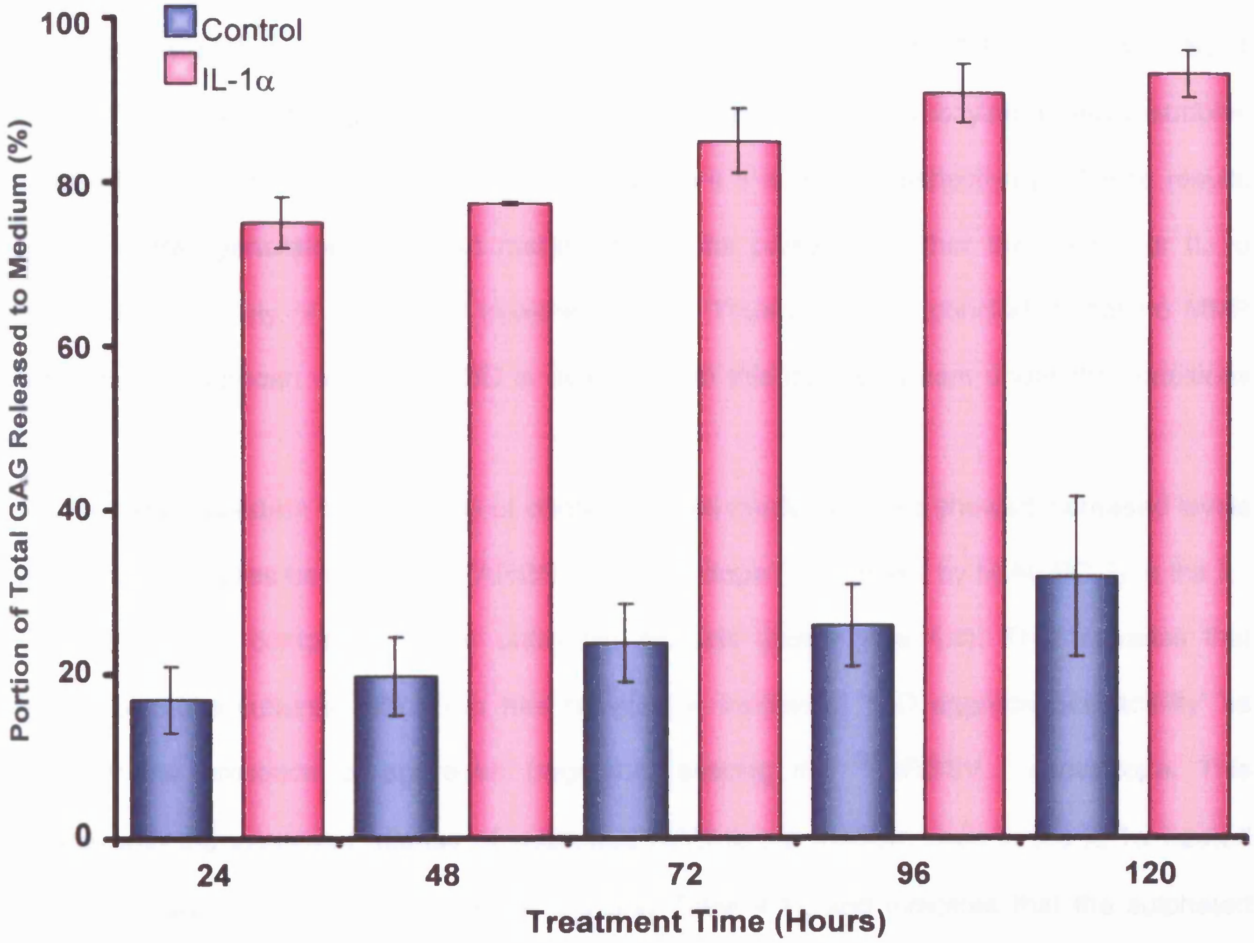


Figure 4.3 Histogram of the mean values for the percentage of the total sulphated GAG released to the experimental medium of control and IL-1α (10ng/ml) treated cultures in the treatment time. Errors shown are standard error. Triplicate plates were treated in three experiments giving a total n of 9.

4.4.2 Analysis of Aggrecan Catabolites by Western Blotting

In order to determine the composition of the aggrecan metabolites in the medium a series of Western blots were carried out, under reducing conditions, using previously characterised M'Abs which specifically recognise neoepitopes resulting from catalytic cleavage within the aggrecan core protein.

Western blot analysis was carried out using M'Abs BC-14 (^{342}FFGV...) and BC-4 (...DIPEN^{341}) to detect MMP-generated aggrecan catabolites in the deglycosylated media samples and guanidine extracts of agarose plugs, (see Figures 4.4 and 4.5), respectively. These results showed no MMP-generated aggrecan metabolites to be present in either the control or IL-1α treated cultures at any of the tested treatment times. Thus it may be concluded that no MMP activity against aggrecan within the IGD is detectable in this culture system under the conditions used.

However, Western blot analysis of deglycosylated media samples showed increased levels of aggrecan catabolites bearing the ^{374}ARGSV... neoepitope (recognised by M'Ab BC-3) in the IL-1α treated cultures compared to the untreated controls (see Figure 4.6). This indicates that treatment of these cultures with IL-1α has resulted in increased 'IGD aggrecanase activity' as defined by the presence of aggrecan fragments bearing the ^{374}ARGSV... neoepitope. This correlates with the increased release of sulphated GAG to the medium seen in the IL-1α treated cultures compared to controls (see Figure 4.3 and Table 4.1), and indicates that the sulphated GAG release seen in these cultures is the result of increased 'aggrecanase activity' within the IGD of the aggrecan core protein. The aggrecan catabolites detected using BC-3 in the IL-1α treated cultures are high molecular weight products in the range of >250 - 150kD. In the shorter treatment times (24 - 48 hours) the higher molecular weight catabolites predominate. With increasing treatment time in the IL-1α cultures the intensity of the lower molecular weight catabolites (~150kD) increases as the intensity of the higher molecular weight metabolites (>250kD) decreases. This data indicates that the aggrecan catabolites, initially resulting from cleavage within the IGD alone, are further degraded at additional sites carboxy-terminal to the IGD to form smaller molecular weight species over time in the presence of IL-1α.

Western blot analysis showed G1 aggrecan catabolites bearing the ...NITEGE^{373} neoepitope (as detected by M'Ab BC-13) to be present in guanidine extracts of agarose plugs at time zero, and in both control and IL-1α treated cultures at all time points investigated (see Figure 4.7). The presence of aggrecanase-generated aggrecan metabolites in untreated cultures at time zero was confirmed by positive immunostaining by Western blot analyses of pre-culture medium with M'Ab BC-3 (data not shown). The presence of both BC-13 and BC-3 immunopositive aggrecan metabolites in untreated cultures at time zero indicated aggrecanases to be involved in the matrix turnover which occurs during the preculture period in the presence of serum.

Following 96 hours of treatment increased levels of immunopositive staining were detected (using M'Ab BC-13) in guanidine extracts of agarose plugs from IL-1α treated cultures compared to controls (see Figure 4.7). Following 120 hours of treatment both the control and IL-1α treated cultures show increased levels of immunopositive staining compared to the levels detected at earlier time points, excluding 96 hours. This data indicates BC-13 staining does not correspond with the BC-3 staining seen in Figure 4.6 i.e. BC-13 positive catabolites do not increase in the IL-1α treated cultures at all time points. The variability detected in staining of Western blots may reflect the poor extractability of proteoglycans using guanidine HCl, as discussed earlier and shown in Table 4.1. In addition, this may be due to loss of the ...NITEGE^{373} neoepitope through trimming by MMPs (Van-Meurs *et al.,* 1999, and Little *et al.,* 2002b). However since no BC-4 staining was detected (see Figure 4.5) it is unlikely that this trimming was due to MMPs in this culture system. Alternatively, cleavage of the aggrecan core protein at the Glu^{373} - Ala^{374} bond within the IGD may result in loss of both aggrecan catabolites to the medium due to the 'looser' nature of the matrix produced by this culture system compared to the matrix present in cartilage explant cultures. Thus both the carboxy-terminal portion bearing the ^{374}ARGSV... neoepitope detected by BC-3 and the amino-terminal portion bearing the ...NITEGE^{373} neoepitope detected by BC-13 could be present in the medium from these cultures. This was confirmed by immunopositive staining on Western blots of deglycosylated media samples probed with M'Ab BC-13 (data not shown).

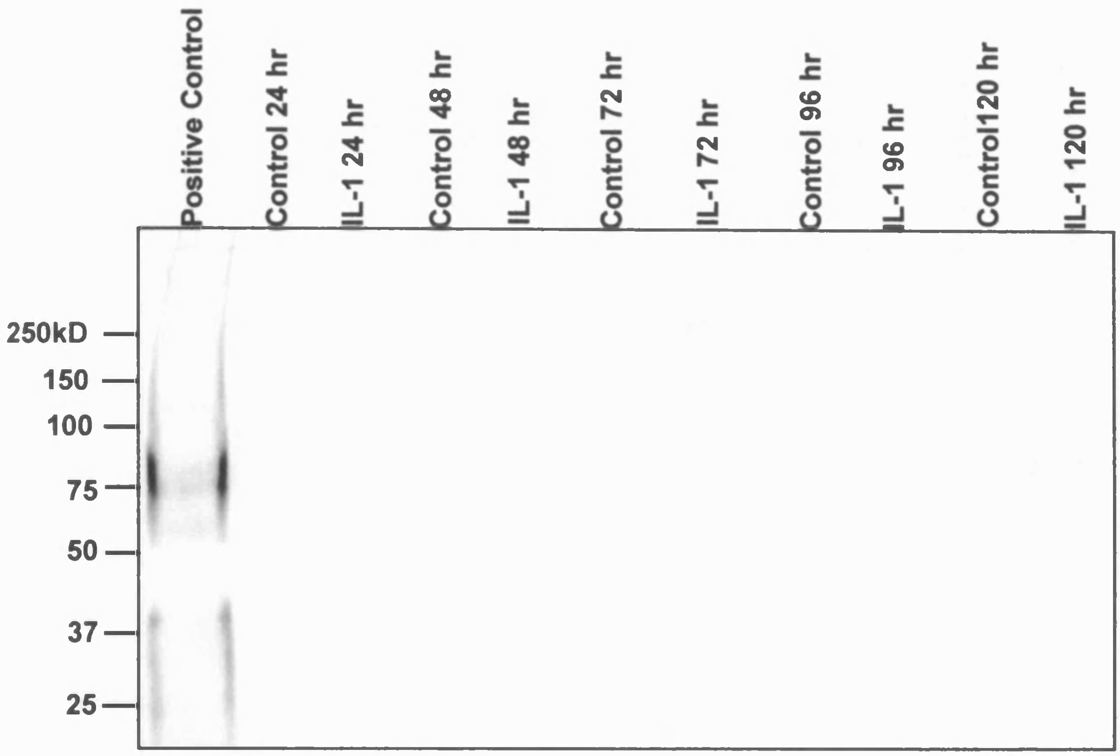


Figure 4.4 Western blot analysis of MMP-generated aggrecan metabolites containing the IGD neoepitope ^{342}FFGV... detected with M'Ab BC-14. Western blot analysis of media samples from cultures treated in the absence (control) or presence of IL-1α (10ng/ml) for 24 - 120 hours (20μg GAG equivalent per lane). The positive control was MMP-13 digested A1D1 (20μg GAG equivalent).

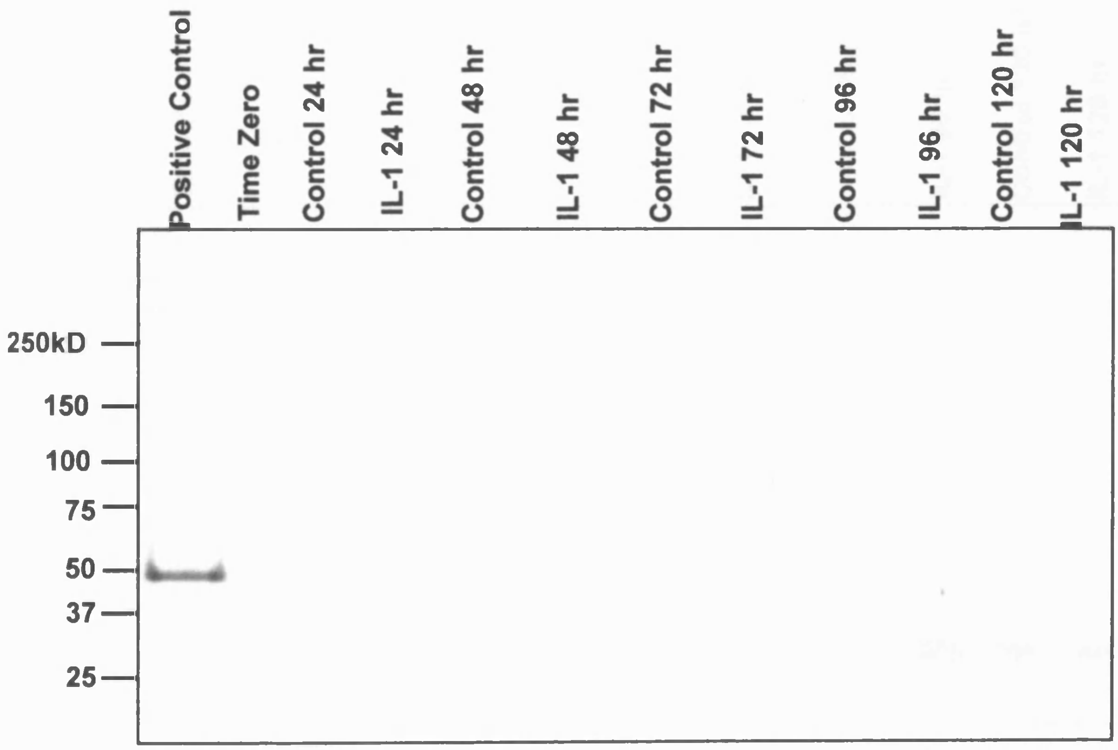


Figure 4.5 Western blot analysis of MMP-generated aggrecan metabolites containing the IGD neoepitope ...DIPEN^{341} detected with M'Ab BC-4. Western blot analysis of guanidine extracts of agarose plugs of cultures at time zero and following treatment in the absence (control) or presence of IL-1α (10ng/ml) for 24 - 120 hours (20μg GAG per lane). The positive control is MMP-13 digested A1D1 (20μg GAG equivalent).

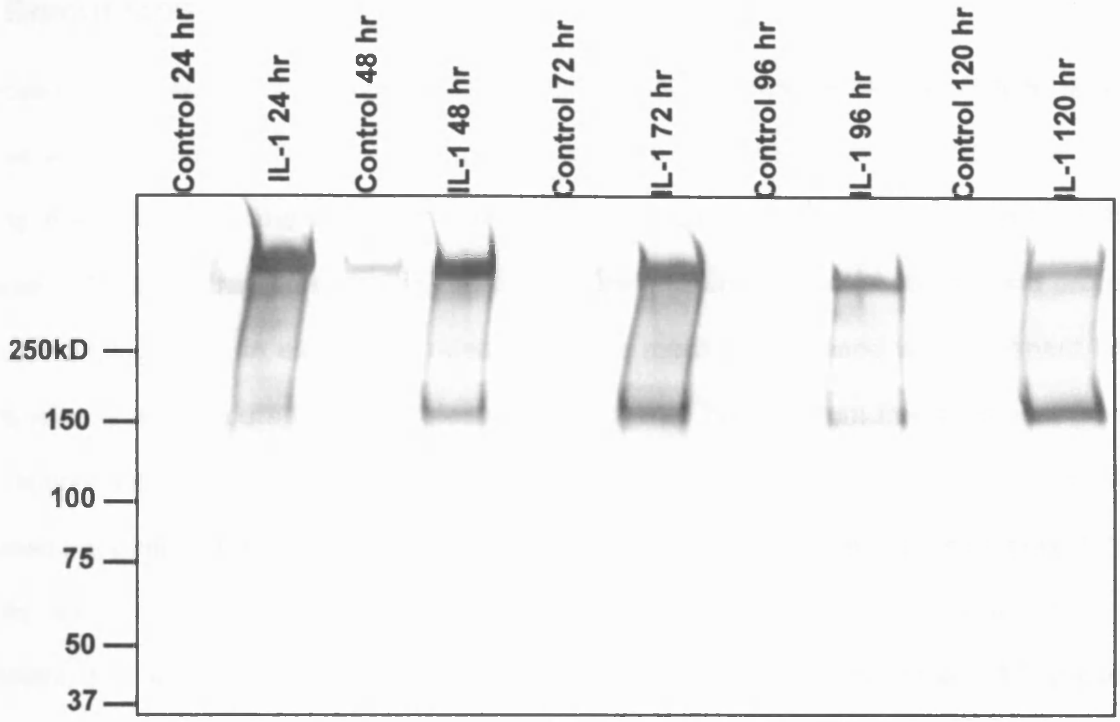


Figure 4.6 Western blot analyses of aggrecanase-generated aggrecan metabolites containing the IGD neoepitope ^{374}ARGSV... detected with M'Ab BC-3. Western blot analysis of media samples from cultures treated in the absence (control) or presence of IL-1α (10ng/ml) for 24 - 120 hours (20μg GAG equivalent per lane).

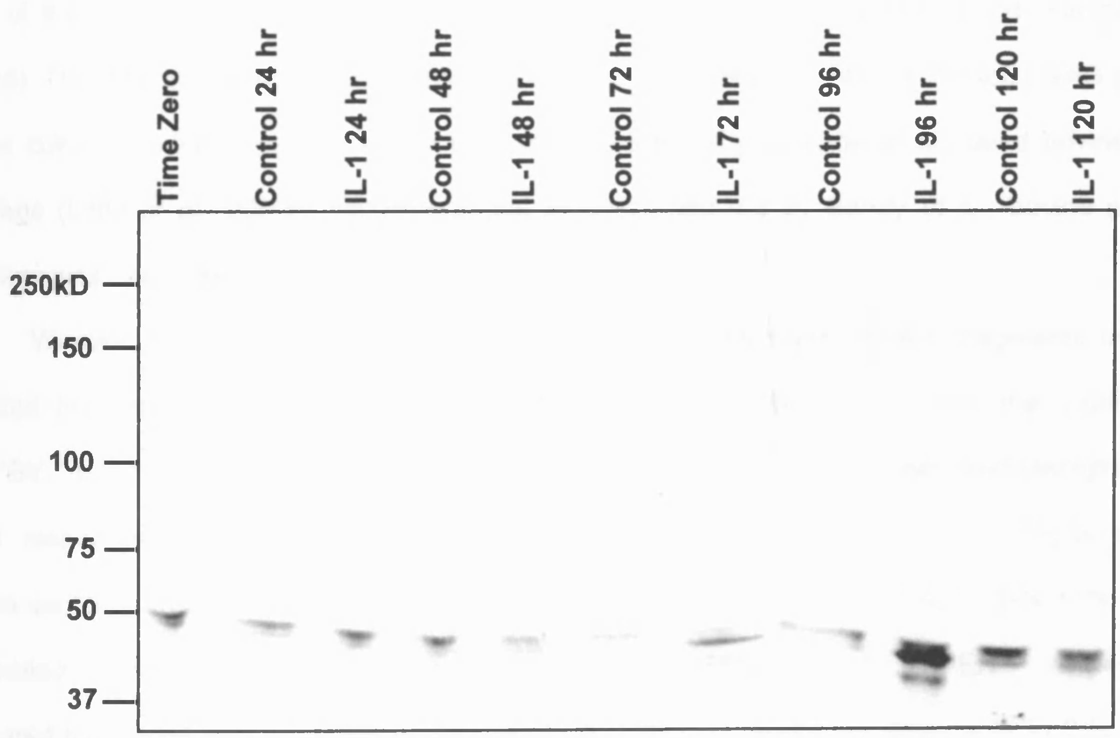


Figure 4.7 Western blot analysis of aggrecanase-generated aggrecan metabolites containing the IGD neoepitope ...NITEGE^{373} detected with M'Ab BC-13. Western blot analysis of guanidine extracts of agarose plugs of cultures at time zero and following treatment in the absence (control) or presence of IL-1α (10ng/ml) for 24 - 120 hours (20μg GAG equivalent per lane).

4.5 Discussion

Agarose cultures were precultured for 21 days to generate an extracellular matrix then washed and treated in serum free medium in the absence (control) or presence of IL-1α for 24 - 120 hours. During this treatment time aggrecan (as determined by the release of sulphated GAG) was released from the extracellular matrix into the culture medium. In control cultures the proportion of the total GAG present in each plate released to the medium increased with treatment time from <20% release at 24 hours to >30% release following 120 hours serum free treatment (see Figure 4.2). In contrast, the addition of IL-1α to these cultures resulted in the percentage of the total GAG released per plate to the medium to be >80% over the same culture period (see Figure 4.2). These results are similar to those previously reported for cartilage explants exposed to the same treatment. For example Little *et al.*, reported release of >80% of the total GAG present from explant cultures treated in the presence of IL-1 for 7 days (Little *et al.*, 2002b). Sandy *et al.*, reported release to the medium of only 36% of the total GAG present from explant cultures treated in serum free medium for 15 days (Sandy *et al.*, 1991a). The addition of IL-1α increased this to 65% of the total GAG being released to the medium over the same culture period (Sandy *et al.*, 1991a). The differences in the culture period required for release of >50% of the total GAG present to the culture medium may be due to the fact that the study by Little *et al.*, used bovine nasal cartilage (Little *et al.*, 2002b) whereas in the study carried out by Sandy *et al.*, bovine articular cartilage was used (Sandy *et al.*, 1991a).

Western blot analyses were carried out in order to determine whether fragments released resulted from cleavage of aggrecan at the two sites within the IGD namely the ...DIPEN^{341}-^{342}FFGV... bond and the ...NITEGE^{373}-^{374}ARGSV... bond (Fosang *et al.*, 1995, and Maniglia *et al.*, 1991, respectively). The absence of immunopositive staining on Western blots of deglycosylated media samples and guanidine extracts probed with M'Abs BC-14 and BC-4, to detect the MMP-generated amino- and carboxy-terminal neoepitopes ^{342}FFGV... and ...DIPEN^{341} respectively, indicated that there was no MMP activity within the IGD of aggrecan detectable in this culture system (see Figures 4.4 and 4.5). This is in contrast to previous reports which have detected MMP-generated aggrecan metabolites in articular cartilage explant cultures treated in serum free medium for 5 days (Fosang *et al.*, 2000). In these explant cultures no increase was detected in the levels of MMP activity against the interglobular domain of aggrecan between cultures treated in the absence or presence of IL-1α for 1-5 days. Therefore the MMP activity against the interglobular domain is unlikely to be involved in aggrecan degradation in response to IL-1α. Little *et al.,* showed increased MMP activity against the interglobular domain of aggrecan following treatment of nasal cartilage explant cultures in the presence of IL-1 for 21 days (Little *et al.,* 2002b). However, activity at the 'aggrecanase site' within the IGD of aggrecan was detected in the explant cultures following only 7 days of culture in the presence of IL-1, whereas MMP activity against the IGD of aggrecan was not detected until 21 days of treatment. In addition MMP activity against the IGD of aggrecan was detected at a later time point than MMP activity against type II collagen. Therefore it would appear that activity of MMPs against the IGD of aggrecan occurs only in very late stage cartilage degradation. In the system used here the treatment period of 120 hours may be insufficient to see any effect of IL-1α on the activity of MMPs for aggrecan. In addition, any background levels of MMP turnover of aggrecan in this culture system appear to be below detection limits.

In contrast, BC-3 and BC-13 positive staining were detected in preculture medium (data not shown) and agarose plugs at time zero (see Figure 4.7), and were further enhanced in IL-1α treated cultures, strongly indicating aggrecanases to be active during the preculture period and treatment regime. The base level of 'aggrecanase activity' detected in the agarose cultures prior to treatment in serum free conditions is significantly increased in the cultures exposed to IL-1α (see Figure 4.6). Immunopositive staining with M'Ab BC-3 indicates that the release of sulphated GAG to the medium, detected by the DMMB assay, was the result of 'aggrecanase activity' within the IGD of the aggrecan core protein. This corresponds with numerous previously published results, which detected increased aggrecanase-generated aggrecan catabolites in the medium of explant cultures treated with IL-1 (Gendron *et al.,* 2003, Hughes *et al.,* 1995, and Arner *et al.,* 1998).

This agarose culture system provides a means of rapidly (24 hours compared to 1 week) evaluating the effects of IL-1α exposure on chondrocyte / cartilage metabolism and thus may be useful for "high throughput" evaluation of drugs (therapeutic agents) on inhibiting this process.

4.6 Summary

- In this chapter the effect of exposure of the aggrecan present in the extracellular matrix of chondrocyte-agarose cultures to the catabolic stimulant IL-1α, was investigated.
- Aggrecan turnover during the preculture period, in the presence of serum, was shown to be due to 'aggrecanase activity' within the IGD of the aggrecan core protein (detected using M'Ab BC-13).
- Treatment of chondrocyte-agarose cultures with IL-1α resulted in increased 'aggrecanase activity' (detected using M'Ab BC-3) and release of sulphated GAG to the culture medium compared to the levels detected in untreated control cultures.
- Thus again the matrix produced by chondrocytes embedded in agarose has been shown to mimic the extracellular matrix of articular cartilage and is therefore a useful model system to investigate the enzymes involved in degradation of the aggrecan present in this matrix. In this chapter these enzymes were shown to be aggrecanases i.e. ADAMTS-4, -5 and -1 (see Sections 1.3.5 and 1.5.2).

Chapter 5: Investigation of ADAMTS-4 and -5 Isoforms Present in Chondrocyte-Agarose Cultures

5.1 Introduction

The extracellular matrix produced by the model culture system of chondrocytes embedded in agarose was investigated in Chapter 3 and shown to have similarities to the matrix of articular cartilage in its proteoglycan composition. In Chapter 4 the effect of exposure of this matrix to the catabolic stimulant IL-1α was investigated. The aggrecan present was degraded by aggrecanases and resulted in the release of aggrecan metabolites some of which bear the interglobular domain neoepitope ^{374}ARGSV... and were highly sulphated (as measured by the DMMB assay).

As the aggrecanases are now known to include the enzymes ADAMTS-4 and -5 (see Figure 5.1), reviewed in the Introduction of this thesis (Chapter 1 Section 1.3.5 and 1.5.2), and are thought to be key players in the degradation of aggrecan in diseases such as osteoarthritis and rheumatoid arthritis, their presence and contribution to the degradation of aggrecan in the culture system of chondrocytes embedded in agarose was investigated.

Little is known of the mechanisms of action of ADAMTS-4 and -5 in cartilaginous extracellular matrices. In order to investigate the synthesis and secretion of these enzymes, commercially available polyclonal antibodies raised against the propeptide and spacer domains of ADAMTS-4 and -5 were purchased, and a new linear epitope monoclonal antibody (M'Ab) Anti-TS-4N which recognises amino acids within the sequence ^{213}FASLSRFV^{220} present at the amino-terminal end of the metalloproteinase domain of ADAMTS-4 (see Figure 5.1), was produced in our laboratory. This sequence is thought to form the amino-terminus of the protein in Furin-cleaved ADAMTS-4 (Molloy *et al.*, 1992, and Tortorella *et al.*, 1999). Purified furin can cleave the proform of ADAMTS-4 *in vitro* within the consensus sequence ^{206}RPRRAKR^{212} (Gao *et al.*, 2002). Furthermore, recombinant mutants of pro-ADAMTS-4 indicated cleavage to occur at three sites within this sequence ^{206}RPRR^{209}, ^{209}RAKR^{212} and ^{211}KR^{212} (Wang *et al.*, 2004).

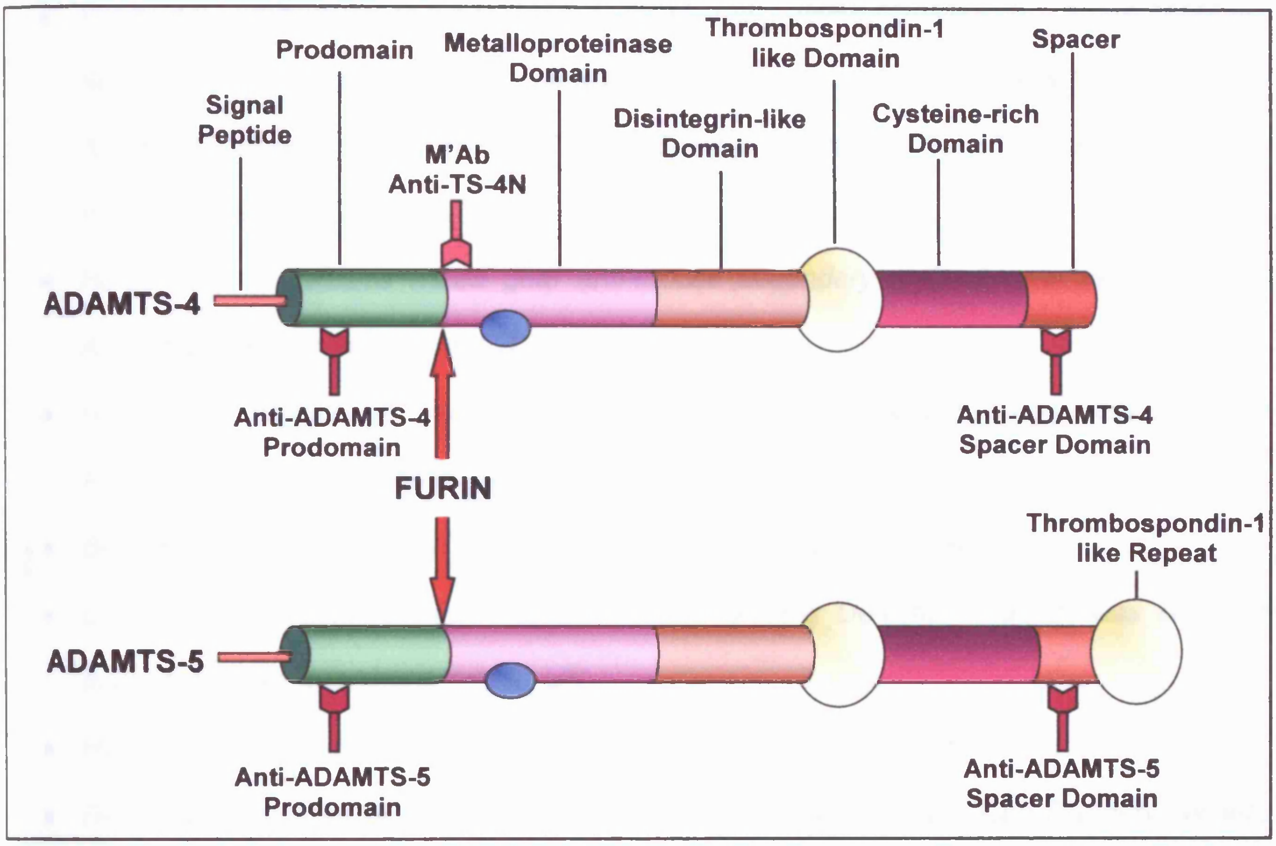


Figure 5.1 Proposed structures of the proforms of ADAMTS-4 and –5 with the Furin cleavage sites indicated by the red arrows. The regions recognised by the commercially available polyclonal antibodies are shown. Anti-ADAMTS-4 prodomain and Anti-ADAMTS-4 Spacer domain recognise ADAMTS-4, and Anti-ADAMTS-5 prodomain and Anti-ADAMTS-5 Spacer domain recognise ADAMTS-5. Also indicated is the newly characterised monoclonal antibody Anti-TS-4N which specifically recognises the sequence ^{213}FASLSRFV^{220} at the amino terminus of the metalloproteinase domain of ADAMTS-4.

5.2 Materials

- *Monoclonal antibody Anti-TS-4N was produced by Dr. Clare Hughes and Dr. Chris Little using methods described previously (Hughes et al., 1995).*
- *Polyclonal antibodies Anti-ADAMTS-4 Prodomain (RP2-ADAMTS-4), Anti-ADAMTS-4 Spacer domain (RP1-ADAMTS-4), Anti-ADAMTS-5 Prodomain (RP2-ADAMTS-5) and Anti-ADAMTS-5 Spacer domain (RP1-ADAMTS-5) were all obtained from Triple Point Biologics Inc., Forest Grove, OR, US.*
- *Horseradish peroxidase linked goat anti-mouse secondary antibody was obtained from Amersham, Buckinghamshire, UK.*
- *Horseradish peroxidase linked goat anti-rabbit secondary antibody was obtained from Amersham, Buckinghamshire, UK.*
- *Blocking agent was obtained from Amersham, Buckinghamshire, UK.*
- *Enhanced Chemiluminescence (ECL) Western blotting Detection reagent was obtained from Amersham, Buckinghamshire, UK.*
- *Hyperfilm ECL was obtained from Amersham, Buckinghamshire, UK.*
- *Recombinant human ADAMTS-4 and –5 were a kind gift from Dr. Carl Flannery, Wyeth, Boston, US.*
- *All other reagents were of laboratory grade and are listed in Chapter 2 Section 2.1 and Chapter 4 Section 4.2.*

5.3 Methods

5.3.1 Specificity of Monoclonal Antibody Anti-TS-4N for Recombinant Human ADAMTS-4 by Western Blotting and Chemiluminescence

- **Optimisation of Western Blotting using Monoclonal Antibody Anti-TS-4N**

Samples (0.5-0.125µg protein per lane) of recombinant human ADAMTS-4 were prepared in Laemmli sample buffer (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed under reducing conditions on 10% SDS-PAGE slab gels in running buffer The gels were then transferred onto Nitrocellulose membrane (0.22µm) in transfer buffer at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis. Membranes were blocked in 5% (w/v) Amersham blocking agent in PBS-T (10mM phosphate, 2.7mM potassium chloride, 137mM sodium chloride pH 7.4 with 0.1% (v/v) tween 20) overnight with constant rocking. The Western blots were washed 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes in PBS-T then incubated in monoclonal antibody (M'Ab) Anti-TS-4N diluted 1:100, 1:200 or 1:500 in 1% (w/v) blocking agent in PBS-T for 1 hour at room temperature with constant rocking. The blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T then incubated in horse-radish peroxidase linked goat anti-mouse secondary antibody diluted 1:1000 in 1% (w/v) blocking agent in PBS-T and incubated on the blots for 1 hour at room temperature with rocking. The blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T before incubation in Amersham ECL developer solution (50:50 solution A and solution B) for 1 minute. The excess liquid was carefully blotted from the membranes with tissue before the membranes were placed between two sheets of clear plastic in a light proof cassette. The Hyperfilm ECL films were exposed for 1-1½ hours before development in an automated developer (Gevamatic 60 from AGFA Gevaert).

- **Specificity of Monoclonal Antibody Anti-TS-4N for Recombinant Human ADAMTS-4**

Recombinant human ADAMTS-4 (1μg) was incubated at 37°C for 20 hours to allow autocatalysis to occur (Flannery *et al.,* 2002). Samples (0.5μg protein per lane) were prepared in Laemmli sample buffer containing 10% (v/v) β-mercaptoethanol and electrophoresed in duplicate on 10% SDS-PAGE gels in running buffer. The gels were then transferred onto Nitrocellulose membrane (0.22μm) in transfer buffer at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis. Membranes were blocked in 5% (w/v) Amersham blocking agent in PBS-T overnight with constant rocking.

Concurrently immunising peptide, sequence ^{213}FASLSRFV^{220} (50μg), was dot blotted onto a piece of Nitrocellulose membrane (0.22μm) and allowed to air dry for 20-30 minutes at room temperature. Optimally diluted Anti-TS-4N (1:200) in 1% (w/v) blocking agent in PBS-T was incubated overnight at 4°C on Nitrocellulose membrane, either blank or dot blotted with immunising peptide.

Western blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T then incubated for 1 hour at room temperature with rocking in the Anti-TS-4N solutions removed from the dot blotted membranes. The blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T, then incubated in horse-radish peroxidase linked goat anti-mouse secondary antibody diluted 1:1000 in 1% blocking agent in PBS-T for 1 hour at room temperature with rocking. The blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T, before incubation in ECL Developer solution (50:50 solution A and solution B) for 1 minute. The excess liquid was carefully blotted from the membranes with tissue before being placed between two sheets of clear plastic in a light proof cassette. The ECL films were exposed for 1-1½ hours before development in an automated developer (Gevamatic 60 from AGFA Gevaert).

5.3.2 Characterisation of Monoclonal Antibody Anti-TS-4N and the Commercially Obtained Polyclonal Antibodies Recognising Protein Domains in Recombinant Human ADAMTS-4 and -5

Recombinant human ADAMTS-4 (1µg) was incubated at 37°C for 20 hours to allow autocatalysis to occur (Flannery *et al.*, 2002). Samples (1µg protein per lane) of recombinant human ADAMTS-4 (autocatalysed) and recombinant human ADAMTS-5 were prepared in Laemmli sample buffer (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed under reducing conditions on 10% SDS-PAGE slab gels in running buffer. The gels were then transferred onto Nitrocellulose membrane (0.22µm) in transfer buffer at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis. Membranes were blocked in 5% (w/v) Amersham blocking agent in PBS-T for a minimum of 1 hour with constant rocking. The Western blots were washed 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes in PBS-T then incubated in optimally diluted monoclonal Anti-TS-4N (1:200) or polyclonal Anti-ADAMTS-4 Prodomain, Anti-ADAMTS-4 Spacer, Anti-ADAMTS-5 Prodomain and Anti-ADAMTS-5 Spacer (all 1:1000) overnight at room temperature with constant rocking. The blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T then incubated in either horse-radish peroxidase linked goat anti-mouse secondary antibody, for the Western blots probed with the M'Ab (Anti-TS-4N), or horse-radish peroxidase linked goat anti rabbit secondary antibody, for the Western blots with the polyclonal antibodies (Anti-ADAMTS-4 Prodomain, Anti-ADAMTS-4 Spacer, Anti-ADAMTS-5 Prodomain or Anti-ADAMTS-5 Spacer). Both secondary antibodies were diluted 1:1000 in 1% (w/v) blocking agent in PBS-T and incubated on the blots for 1 hour at room temperature with rocking. The blots were washed, 2 x 5 minutes, 1 x 15 minutes and 2 x 5 minutes, in PBS-T, before incubation in Amersham ECL developer solution (50:50 solution A and solution B) for 1 minute. The excess liquid was carefully blotted from the membranes with tissue before the membranes were placed between two sheets of clear plastic in a light proof cassette. The Hyperfilm ECL films were exposed for 1-1$^1/_2$ hours before development in an automated developer (Gevamatic 60 from AGFA Gevaert).

5.3.3 Silver Stain of Recombinant Human ADAMTS-5

Recombinant human ADAMTS-5 (1µg protein) was prepared in Laemmli sample buffer (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed under reducing conditions on 10% SDS-PAGE slab gels in running buffer. The gel was prefixed in 30% (v/v) ethanol and 10% (v/v) acetic acid in MilliQ™ water overnight with rocking. The gel was rinsed 2 x 10 minutes in 10% (v/v) ethanol in MilliQ™ water, then 3 x 10 minutes in MilliQ™ water before soaking in 5µg/ml dithiothreitol (DTT) for 30 minutes. The DTT solution was poured off and without rinsing 0.1% (w/v) silver nitrate solution was added for 30 minutes. The gel was rinsed once with a small volume of MilliQ™ water, then twice with 100ml developer (50µl 37% (v/v) formaldehyde in 100ml 3% (w/v) sodium carbonate, anhydrous) until the desired level of staining was obtained. Staining was stopped by addition of exactly 5ml of 2.3M citric acid to the developer and incubation for 10 minutes with rocking. The gel was washed thoroughly with several changes of MilliQ™ water.

5.3.4 Western Blot Analysis of ADAMTS-4 and ADAMTS-5 Isoforms Present in Media Samples and Detergent Extracts of Agarose Plugs

Heparin and zinc chelator column eluents or detergent extracts of agarose plugs (prepared in Chapter 4 Section 4.3.1 and Chapter 2 Sections 2.2.9 and 2.2.10) (all 50µl per lane) along with recombinant human ADAMTS-4 and –5 (0.5µg per lane) were prepared in Laemmli sample buffer containing 10% (v/v) β-mercaptoethanol and electrophoresed in duplicate on 10% SDS-PAGE gels in running buffer. The gels were then transferred onto Nitrocellulose membrane (0.22µm) in transfer buffer at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis as described in Chapter 5 Section 5.3.2.

5.3.5 Analysis of 'Aggrecanase Activity' of Heparin and Zinc Chelator Bound Media Samples from IL-1α Treated Cultures Against the IGD of Purified Aggrecan (A1D1)

Purified aggrecan (A1D1) was prepared as described in Chapter 2 Section 2.2.6. To aliquots of A1D1 (100μg GAG equivalent) was added 300μl Heparin-Sepharose or Zinc Chelator column eluents (see Section 5.3.6) with 1/10 volume 10x buffer (20mM tris, 100mM sodium chloride, 10mM calcium chloride, pH 7.5 with 2.5% (v/v) triton) in the absence or presence of M'Ab Anti-TS-4N (500μl) or control M'Ab 70.6 Anti-Decorin (500μl). The digestions were incubated at 37°C for 24 hours before precipitation of the chondroitin sulphate bearing aggrecan fragments using cetylpyridinium chloride (CPC).

To the digestion mixtures was added a 1/10-volume 10x sodium sulphate buffer (0.5M sodium sulphate). Cetylpyridinium chloride (CPC) solution (10% [w/v] in MilliQ™ water) was added drop wise at room temperature until a precipitate formed. The precipitate was spun down at room temperature at 2000 x g for 5 minutes and the supernatant discarded. The pellets were washed twice in 0.05% (w/v) CPC solution (4ml) to remove any sodium ions then resuspended in 0.5ml 80% (v/v) propan-1-ol in water. To the mixtures was added 100μl saturated sodium acetate solution (1g/8ml in MilliQ™ water), 1 drop acetic acid and 3ml cold ethanol and they were left to precipitate overnight at 4°C. The precipitate was spun down at 2000 x g for 5 minutes, the supernatant discarded and the pellets dried under a vacuum. The pellets were resuspended in 0.1M tris acetate pH 6.5 and deglycosylated, dialysed and lyophilised on a speedvac as described in Chapter 2 Section 2.8. Following this the samples were reconstituted, in Laemmli sample buffer (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed under reducing conditions on 4-12% Tris Glycine gels, transferred and subjected to Western blot analysis with M'Ab BC-3 as described in Chapter 2 Section 2.2.8.

5.4 Results

5.4.1 Optimisation of Western Blot Analysis of Human Recombinant ADAMTS-4 using Monoclonal Antibody Anti-TS-4N

In order to investigate the possible isoforms of ADAMTS-4 present in various culture systems a monoclonal antibody (M'Ab) which recognises amino acids within the sequence ^{213}FASLSRFV^{220} present at the amino-terminal end of the metalloproteinase domain of ADAMTS-4, was produced. This sequence is thought to form the amino-terminus of the protein in Furin-activated ADAMTS-4 (Molloy *et al.,* 1992, Gao *et al.,* 2002, Wang *et al.,* 2004, and Tortorella *et al.,* 1999). A number of hybridoma clones were obtained from immunisation of one mouse with the ovalbumen-conjugated peptide (^{213}FASLSRFV^{220}), a clone designated Anti-TS-4N reacted strongly in an Enzyme Linked Immunosorbant Assay (ELISA) with the immunising peptide, but showed no reactivity with unrelated peptide conjugates nor with the carrier protein (results not shown).

In order to determine the optimal quantities of recombinant human ADAMTS-4 used for peptide inhibition analysis of the new M'Ab Anti-TS-4N a series of Western blots of samples of human recombinant ADAMTS-4 (0.5-0.125μg protein per lane) were probed with a variety of dilutions (1:100-1:500) of the antibody.

The results show that detection of 0.5μg human recombinant ADAMTS-4 was achieved at all antibody dilutions used. The detection of 0.25μg of protein was attainable with a 1:200 antibody dilution, whilst 0.125μg was detected using a 1:100 antibody dilution. For much of the subsequent analysis an antibody dilution of 1:100-1:200 was used, dependent on the time of antibody incubation.

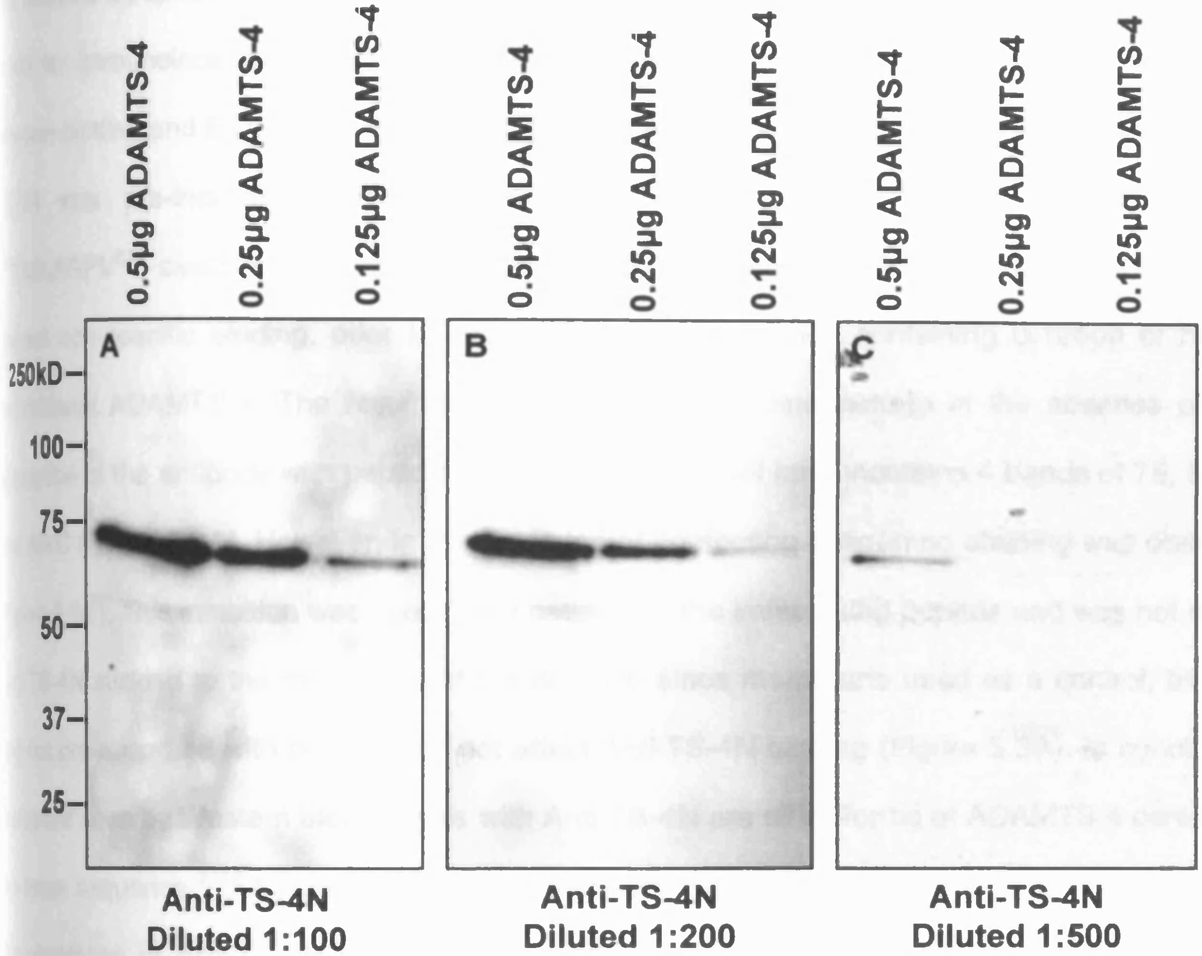


Figure 5.2 Optimisation of Western blot analysis of recombinant human ADAMTS-4 with monoclonal antibody Anti-TS-4N. Samples of recombinant human ADAMTS-4 (0.5, 0.25 and 0.125µg protein) were subjected to Western blot analysis using monoclonal antibody Anti-TS-4N (A) Diluted 1:100, (B) Diluted 1:200 and (C) Diluted 1:500.

5.4.2 Specificity of Monoclonal Antibody Anti-TS-4N for Recombinant Human ADAMTS-4 Using Peptide Inhibition Analysis

In order to confirm the specificity of the monoclonal antibody Anti-TS-4N for ADAMTS-4 in Western blot analysis a peptide inhibition was carried out. The optimal antibody dilution was determined as 1:100 for immunolocalisation of 0.125µg autocatalysed human recombinant ADAMTS-4 using Western blotting and ECL as the detection method (see Figure 5.2). Hence a 1:100 dilution of Anti-TS-4N was pre-incubated with nitrocellulose membranes, dot blotted with or without ^{213}FASLSRFV^{220} ovalbumen conjugated peptide (50µg protein) and subsequently blocked to prevent non-specific binding, prior to incubation with membranes containing 0.125µg of human recombinant ADAMTS-4. The results shown in Figure 5.3 demonstrate in the absence of pre-absorption of the antibody with peptide conjugate, Anti-TS-4N immunostains 4 bands of 75, 55, 45 and 40kD (Figure 5.3A). However, in the presence of competing antigen no staining was observed (Figure 5.3B). This inhibition was specifically caused by the immunising peptide and was not due to Anti-TS-4N sticking to the membrane of the dot blot, since membrane used as a control, blocked and not pre-absorbed with peptide, did not affect Anti-TS-4N binding (Figure 5.3A). In conclusion, the bands seen by Western blot analysis with Anti-TS-4N are all isoforms of ADAMTS-4 containing the linear sequence ^{213}FASLSRFV^{220} at the amino-terminal end of their metalloproteinase domain. Pre-absorption of Anti-TS-4N with the immunising peptide conjugate results in the loss of this staining (Figure 5.3B).

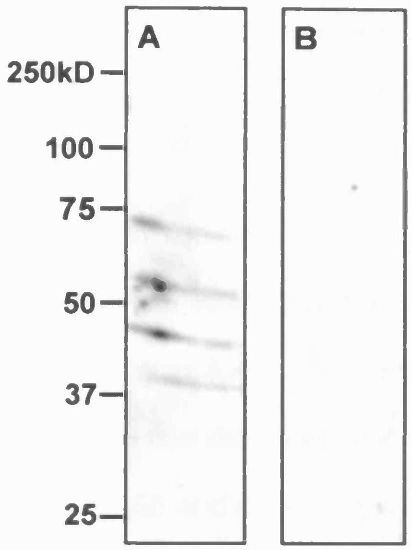


Figure 5.3 Specificity of M'Ab Anti-TS-4N for recombinant human ADAMTS-4 using peptide inhibition analysis. Samples of autocatalysed recombinant human ADAMTS-4 (0.125μg protein per lane) were subjected to Western blot analysis using M'Ab Anti-TS-4N. Anti-TS-4N (diluted 1:100) was preincubated on (A) BSA blocked membrane and (B) Membrane dot blotted with ovalbumen-conjugated immunising peptide (^{213}FASLSRFV^{220}) prior to BSA blocking.

5.4.3 Characterisation of ADAMTS-4 and –5 Mono- and Polyclonal Antibodies Using Recombinant Human ADAMTS-4 and -5 Protein Preparations

Samples of recombinant human ADAMTS-4 and –5 (0.5μg protein per lane), which had been allowed to undergo autocatalysis, were electrophoresed under reducing conditions on 10% SDS-PAGE slab gels and subjected to Western blot analysis using the mono- and polyclonal antibodies recognising various domains of ADAMTS-4 and –5. The results of these analyses are shown in Figure 5.4.

The polyclonal antibody raised against the amino-terminal prodomain of ADAMTS-4 did not detect any bands of ADAMTS-4 protein indicating that none of the recombinant human protein is released with its prodomain intact (Figure 5.4A). The newly characterised M'Ab, Anti-TS-4N, which recognises the amino-terminus of the metalloproteinase domain of ADAMTS-4 detected a series of bands of ADAMTS-4 protein at ~75, 55 and 45kD (Figure 5.4B). The predicted molecular weight of Furin-activated human ADAMTS-4 is 67.9kD, therefore it is assumed that the 75kD isoform represents the mature Furin-activated form of ADAMTS-4 and the smaller isoforms result from autocatalysis of the enzyme within its carboxy-terminal domains (Flannery *et al.*, 2002). Indeed, the 55 and 45kD isoforms have been previously described and shown to result from carboxy-terminal truncation of the protein (Flannery *et al.*, 2002). However, interestingly, the polyclonal antibody raised against a peptide sequence in the carboxy-terminal spacer domain of ADAMTS-4 recognised the same three isoforms of ADAMTS-4 protein as Anti-TS-4N (Figure 5.4C). As expected there was no immunoreactivity seen with any of these three antibodies against the recombinant human ADAMTS-5.

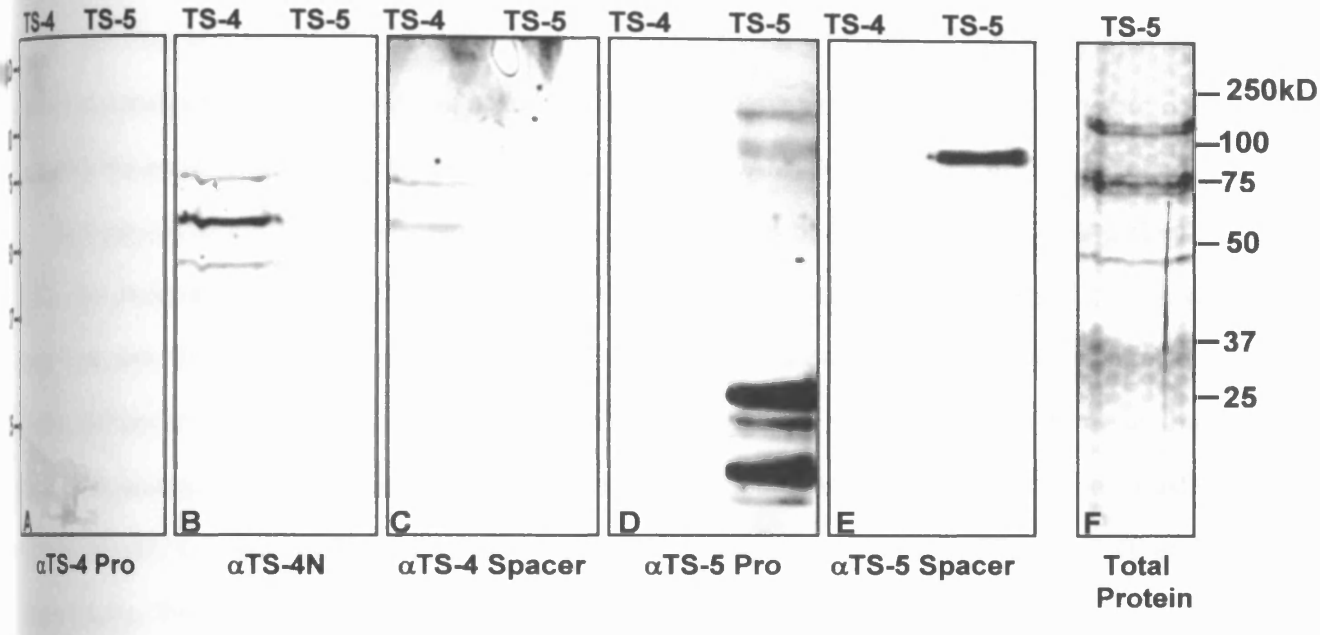


Figure 5.4 Western blot analyses of recombinant human ADAMTS-4 (TS-4) and ADAMTS-5 (TS-5) (0.5μg protein per lane). Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (αTS-4 Pro), (B) The amino-terminus of the metalloproteinase domain of ADAMTS-4 (αTS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (αTS-4 Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (αTS-5 Pro), (E) The spacer domain of ADAMTS-5 (αTS-5 Spacer) and (F) Silver stain for total protein.

The polyclonal antibody raised against the amino-terminal prodomain of ADAMTS-5 detected a series of bands in the recombinant ADAMTS-5 protein preparation at 105, 90, 30, 27, 20 and 15kD (Figure 5.4D). The predicted molecular weight of the zymogen form of human ADAMTS-5 is 101.7kD, this strongly suggests that the isoforms detected at 105 and 90kD may represent an intact zymogen form of the enzyme. The difference between the 105 and 90kD isoforms may be the result of variable glycosylation of the enzyme. The smaller ADAMTS-5 isoforms detected are assumed to be the result of extracellular removal of the prodomain by enzyme autocatalysis or another enzyme present in the culture system. The small isoforms are not detected by the polyclonal antibody to the spacer domain of ADAMTS-5 (Figure 5.4E).

The polyclonal antibody raised against the spacer domain of ADAMTS-5 detected a single band in the recombinant ADAMTS-5 protein preparation at ~90kD (Figure 5.4E). This band corresponds with the 90kD band detected by the polyclonal antibody raised against the amino-terminal prodomain of ADAMTS-5 thus this isoform may be the intact zymogen form of the enzyme. Interestingly, the polyclonal antibody to the spacer domain did not detect the 105kD isoform of ADAMTS-5 detected by the antibody to the prodomain of the enzyme (Figures 5.4E and D, respectively). This may indicate masking of the spacer domain epitope in this isoform.

The predicted molecular weight of Furin-activated human ADAMTS-5 is 73.6kD, therefore intriguingly, the antibodies raised against ADAMTS-5 did not detect an isoform corresponding to the Furin-activated form in the samples of recombinant protein. As expected there was no immunoreactivity seen with either of these two antibodies against the recombinant ADAMTS-4.

Silver staining for total protein present in the preparation of human recombinant ADAMTS-5 detected bands at 105, 75, 45kD and between 20-35kD (Figure 5.4F). The 75 and 45kD isoforms of ADAMTS-5 were not detected by either of the antibodies raised against ADAMTS-5. The 75kD isoform detected may be the active Furin processed form of the enzyme (predicted human molecular weight 73.6kD).

5.4.4 Western Blot Analysis of Detergent Extracted Agarose Plugs at Time Zero

In order to establish whether ADAMTS-4 and -5 are sequestered in the extracellular matrix of the model culture system detergent extraction of the agarose plugs was carried out at time zero prior to treatment in serum free conditions. The detergent extracts may also contain intracellular and membrane-bound / associated proteins. The extracts were electrophoresed under reducing conditions on 10% SDS-PAGE slab gels and subjected to Western blot analysis using the series of commercially available polyclonal antibodies (characterised in Section 5.4.3) and the M'Ab Anti-TS-4N.(characterised in Sections 5.4.1, 5.4.2 and 5.4.3). The results are shown in Figure 5.5. Interestingly these analyses show multiple isoforms of ADAMTS-4 and -5 to be present. The molecular weight of the ADAMTS-4 and -5 isoforms detected in detergent extracts of agarose plugs from chondrocyte-agarose cultures differ from those described in Section 5.4.3 for the recombinant enzymes. This may be due to species variation as the predicted molecular weights and recombinant protein preparations used were human and the chondrocyte-agarose cultures used porcine articular chondrocytes.

The ADAMTS-4 and -5 isoforms extracted from agarose plugs were potentially intracellular or membrane-bound / associated, or had been sequestered in the extracellular matrix in the agarose plugs during the 21 day preculture period in the presence of serum.

All of the antibodies raised against the domain-sequences in ADAMTS-4 detected an immunopositive band at 100kD (Figure 5.5 A-C). The 100kD bands detected by the anti-ADAMTS-4 antibodies may represent the intact zymogen form of the enzyme (human predicted molecular weight 90.2kD). Alternatively, it may represent a variety of cassette forms of the enzyme associated with other intracellular molecules or extracellular matrix components. The three anti-ADAMTS-4 antibodies also detect bands at 75 and 55kD as well as a broad band at ~60kD (Figures 5.5 A-C). The 75 and 60kD bands detected by M'Ab, Anti-TS-4N, (Figure 5.5B) and the polyclonal antibody to the spacer domain of ADAMTS-4 (Figure 5.5C) may represent intact Furin-activated forms of ADAMTS-4 (human predicted molecular weight 67.9kD). The polyclonal antibody to the prodomain of ADAMTS-4 also detected an immunopositive band at 250kD, which may correspond to ADAMTS-4 associated with extracellular matrix components such as fibronectin (Hashimoto *et al.*, 2004) or intracellular molecules.

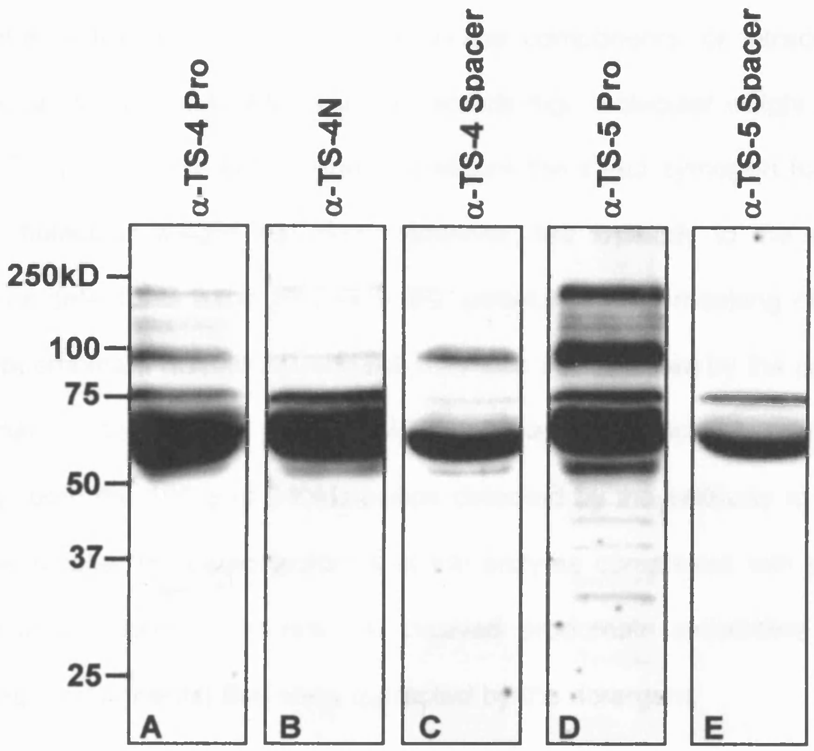


Figure 5.5 Western blot analyses of ADAMTS-4 and –5 present in detergent extracts of agarose plugs (30μl per lane) at time zero following 21 days preculture. Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (Anti-TS-4 Pro), (B) The amino-terminus of the metalloproteinase domain of ADAMTS-4 (Anti-TS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (Anti-TS-4 Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (Anti-TS-5 Pro) and (E) The spacer domain of ADAMTS-5 (Anti-TS-5 Spacer).

The polyclonal antibodies raised against sequences in ADAMTS-5 both detect bands at 75 and 60kD (Figures 5.5D and E). Either of the isoforms detected by the antibody to the spacer domain of ADAMTS-5 (Figure 5.5E) may correspond to the Furin processed active form of the enzyme (predicted human molecular weight 73.6kD). The 60 and 75kD bands detected by the antibody to the prodomain may be smaller carboxy-terminally truncated isoforms of ADAMTS-5 perhaps associated with other extracellular matrix or membrane components, or intracellular molecules. The antibody to the prodomain of ADAMTS-5 also detects high molecular weight bands at 100 and 240kD (Figure 5.5D). The 100kD isoform may represent the intact zymogen form of ADAMTS-5 (predicted human molecular weight 101.7kD). However, the antibody to the spacer domain of ADAMTS-5 does not detect this band (Figure 5.5E), possibly due to masking of the epitope. The 105kD isoform of recombinant human ADAMTS-5 was also not detected by the polyclonal antibody to the spacer domain of the protein presumably due to epitope masking (Section 5.4.3, Figure 5.4E). Alternatively, both the 100 and 240kD bands detected by the antibody to the prodomain of ADAMTS-5 may be smaller truncated isoforms of the enzyme complexed with other extracellular matrix or membrane components, or residual cleaved prodomain associated with intracellular molecules (e.g. Golgi components) that were extracted by the detergent.

5.4.5 Western Blot Analysis of ADAMTS-4 and -5 Isoforms Present in Detergent Extracts of Agarose Plugs Following Treatment in the Presence or Absence of IL1-α

To determine the effects of culture in serum free conditions, with or without stimulation by IL-1α, on the isoforms of ADAMTS-4 and -5 seen in detergent extracts of agarose plugs the extracts were analysed by Western blotting using a panel of antibodies recognising different domains of the enzymes after each time point of the experimental period (see Chapter 4 Section 4.3.1).

Western blot analyses of detergent extracts of agarose plugs from control and IL-1α treated cultures showed a similar banding pattern to that present in the extracts of cultures taken at time zero (Figures 5.6 and 5.5, respectively). No discernable differences were detectable between the time points tested, therefore the results presented in this thesis are only those from cultures treated in the presence or absence of IL-1α for 96 hours (Figure 5.6).

No differences were detectable between control and IL-α treated cultures in the higher molecular weight isoforms present (Figure 5.6). All of the antibodies raised against sequences in ADAMTS-4 detected bands at 100, 75 and 55kD as well as a broad band ~60kD (Figures 5.6 A-C). The polyclonal antibody to the prodomain of ADAMTS-4 also identified an immunopositive band at 250kD (Figure 5.6A). The polyclonal antibodies raised against sequences in ADAMTS-5 both detected bands at 75 and 60kD (Figures 5.6 D and E). The antibody to the prodomain of ADAMTS-5 also detected high molecular weight bands at 240 and 100kD (Figure 5.6D). All of these bands are discussed in detail in Section 5.4.4.

The antibody raised against the prodomain of ADAMTS-5 detected a 32kD band in detergent extracts of agarose plugs from control cultures, which was absent from extracts of agarose plugs from cultures treated with IL-1α (Figure 5.6D). The antibody raised against the spacer domain of ADAMTS-5 detected a 32kD band in detergent extracts of agarose plugs from IL-1α treated cultures which was absent from extracts of agarose plugs from control cultures (Figure 5.6E). These smaller isoforms of ADAMTS-5 may result from catalysis of the enzyme.

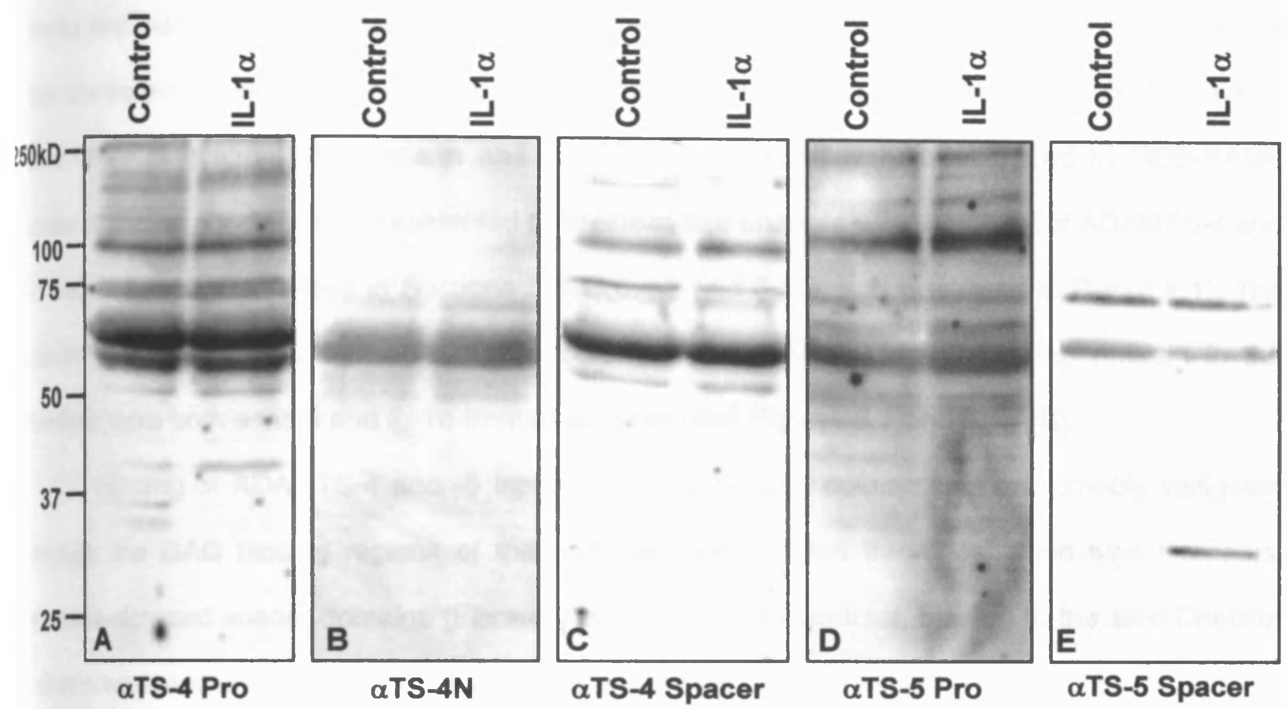


Figure 5.6 Western blot analyses of ADAMTS-4 and -5 isoforms present in detergent extracts of agarose plugs (30μl per lane) of chondrocyte-agarose cultures treated in serum free medium in the absence (control) or presence of IL-1α (10ng/ml) for 96 hours. Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (αTS-4 Pro), (B) The amino-terminus of the metalloproteinase domain of ADAMTS-4 (αTS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (αTS-4 Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (αTS-5 Pro) and (E) The spacer domain of ADAMTS-5 (αTS-5 Spacer).

5.4.6 Western Blot Analysis of ADAMTS-4 and -5 Isoforms Present in the Culture Medium from Control and IL-1α Treated Chondrocyte-Agarose Cultures

Media was harvested and partially purified via passage over a Heparin-Sepharose column where the non-bound fraction from the Heparin-Sepharose column was then subsequently passed over a Zinc Chelator column. The heparin and zinc-binding fractions were fractionated by SDS-PAGE under reducing conditions and subjected to Western blot analysis with the panel of ADAMTS-4 and -5 antibodies (characterised in Sections 5.4.1, 5.4.2 and 5.4.3, and illustrated in Figure 5.1). The resulting Western blots showed multiple isoforms of ADAMTS-4 and –5 to be present in the medium from both control and IL-1α treated cultures (see Figures 5.7 and 5.8 A-E).

Binding of ADAMTS-4 and -5 isoforms to the heparin column was presumably instigated through the GAG binding regions of the enzymes such as the thrombospondin type I repeats, cysteine-rich and spacer domains (Flannery *et al.*, 2002). In contrast, binding to the Zinc-Chelator column was mediated via the metalloproteinase domains of the enzymes.

The molecular weight of the ADAMTS-4 and -5 isoforms detected in media samples from chondrocyte-agarose cultures differ from those described in Section 5.4.3 for the recombinant enzymes. This may be due to species variation as the predicted molecular weights and recombinant protein preparations used were human and the chondrocyte-agarose cultures used porcine articular chondrocytes.

- **Zinc Chelator Bound Media Fractions**

Media Samples Partially Purified via Passage over Heparin-Sepharose and Bound by a Zinc Chelator Column

The zinc chelator bound isoforms of ADAMTS-4 and –5 detected showed no differences between the time points tested, therefore the data is shown for cultures treated in the presence or absence of IL-1α for 96 hours (Figure 5.7) as this is representative of the results obtained for all time points. Staining revealed a complex pattern of bands using the polyclonal antibodies to the prodomains of ADAMTS-4 and -5 (Figures 5.7 A and D, respectively), with more simplistic patterns appearing with the M'Ab Anti-TS-4N and the polyclonal antibodies to the spacer domains of ADAMTS-4 and -5 (Figures 5.7 B, C and E, respectively).

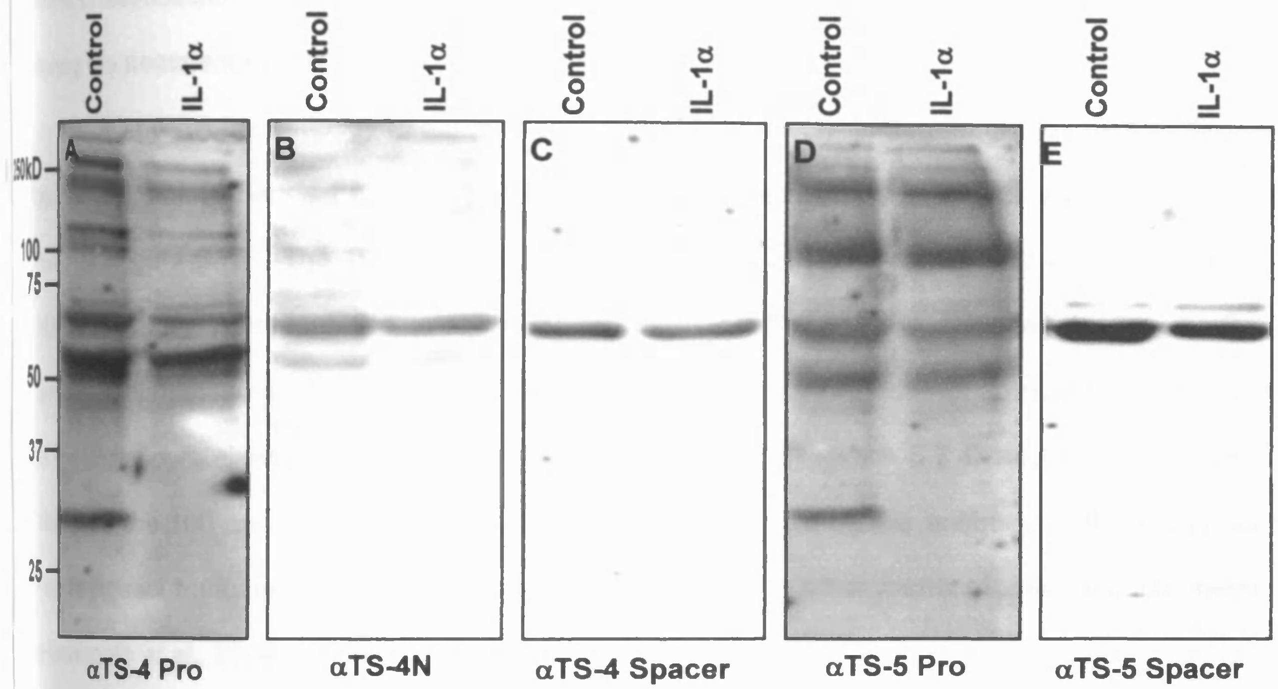


Figure 5.7 Western blot analyses of ADAMTS-4 and -5 isoforms present in media fractions bound to Zinc Chelator column and eluted in 35mM imidazole (50μl per lane) from 21 day chondrocyte-agarose cultures treated in serum free medium in the absence (control) or presence of IL-1α (10ng/ml) for 96 hours. Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (αTS-4 Pro), (B) The amino-terminus of the metalloproteinase domain of ADAMTS-4 (αTS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (αTS-4 Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (αTS-5 Pro) and (E) The spacer domain of ADAMTS-5 (αTS-5 Spacer).

A predominant ~70kD zinc chelator bound isoform of ADAMTS-4 was detected by all of the antibodies raised against different domains of the enzyme in control and IL-1α treated cultures (Figures 5.7 A, B and C). Immunostaining with all three antibodies strongly suggests multiple forms of the enzyme are represented within the relatively broadly stained band. The band may contain a proportion of Furin-cleaved ADAMTS-4 (predicted human molecular weight 67.9kD), as well as cassette forms of prodomain, and carboxy or metalloproteinase domains or combinations there of, alone or associated with other matrix molecules which have been released into the culture medium during the treatment period.

The polyclonal antibody to the prodomain of ADAMTS-4 detected numerous high molecular weight zinc bound bands between 250 and 100kD in medium from control and IL-1α treated cultures (Figure 5.7A). The 100kD band detected by the polyclonal antibody to the prodomain of ADAMTS-4 may represent the intact zymogen form of the enzyme released to the medium (predicted human molecular weight 90.2kD). However, this isoform was not detected by antibodies to the metalloproteinase or spacer domains of ADAMTS-4 (Figures 5.7 B and C, respectively). Therefore the 100 and 250kD isoforms of ADAMTS-4 detected by the antibody to the prodomain may represent truncated forms of the enzyme associated with other matrix proteins e.g. fibronectin (Hashimoto *et al.*, 2004).

Both antibodies to the pro- and metalloproteinase domains of ADAMTS-4 detected a zinc chelator bound band of 55kD in medium from control and IL-1α treated cultures (Figures 5.7 A and B). The polyclonal antibody to the prodomain of ADAMTS-4 detected a ~30kD band in zinc chelator bound samples of medium from control cultures which was absent from IL-1α treated cultures (Figure 5.7B). These smaller isoforms of ADAMTS-4 may result from enzyme catalysis.

The predominant ~70kD zinc chelator bound isoform of ADAMTS-5 is detected by both the antibodies to the pro- and spacer domains of the enzyme in media samples from control and IL-1α treated cultures (Figures 5.7 D and E). The ~70kD isoform may in part represent the Furin-cleaved form of the enzyme (predicted human molecular weight 73.6kD).

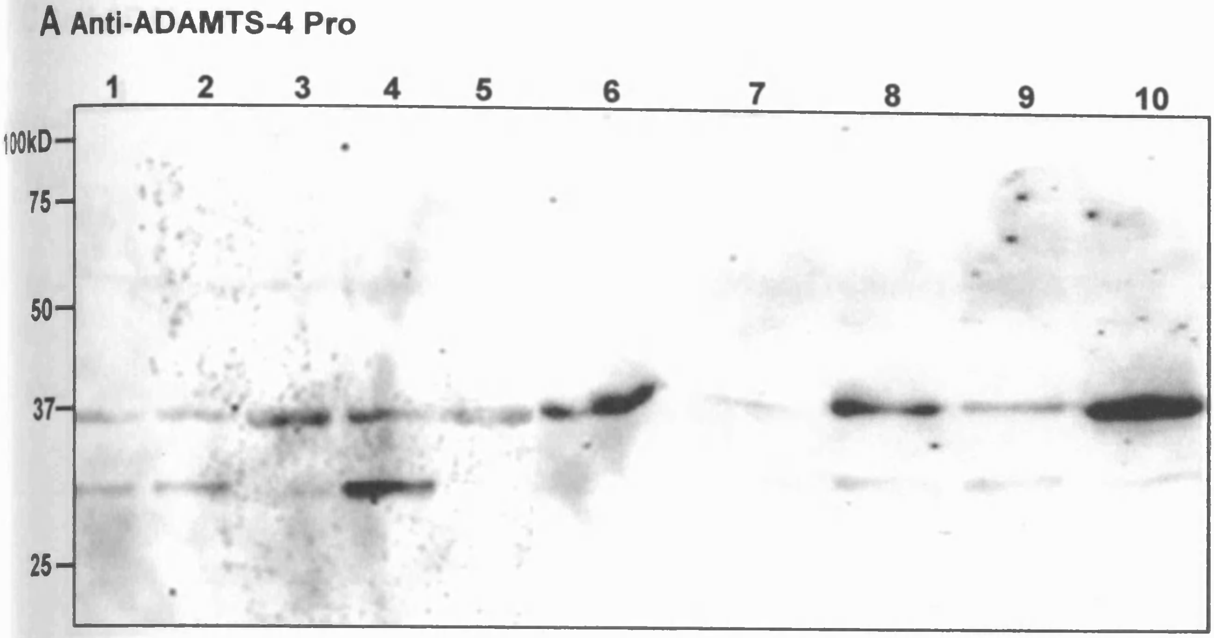
The polyclonal antibody to the prodomain of ADAMTS-5 detected high molecular weight zinc chelator bound bands, at 250 and 100kD, in media from control and IL-1α treated cultures at all time points tested (Figures 5.7D and E). The 100kD isoform of ADAMTS-5 may correspond to the zymogen form of the enzyme (predicted human molecular weight 101.7kD), however the antibody raised against the spacer domain of ADAMTS-5 does not detect this isoform. Therefore both the 100 and 250kD isoforms of ADAMTS-5 detected by the antibody to the prodomain of the enzyme may correspond to smaller isoforms complexed with other matrix proteins e.g. fibronectin (Hashimoto *et al.*, 2004).

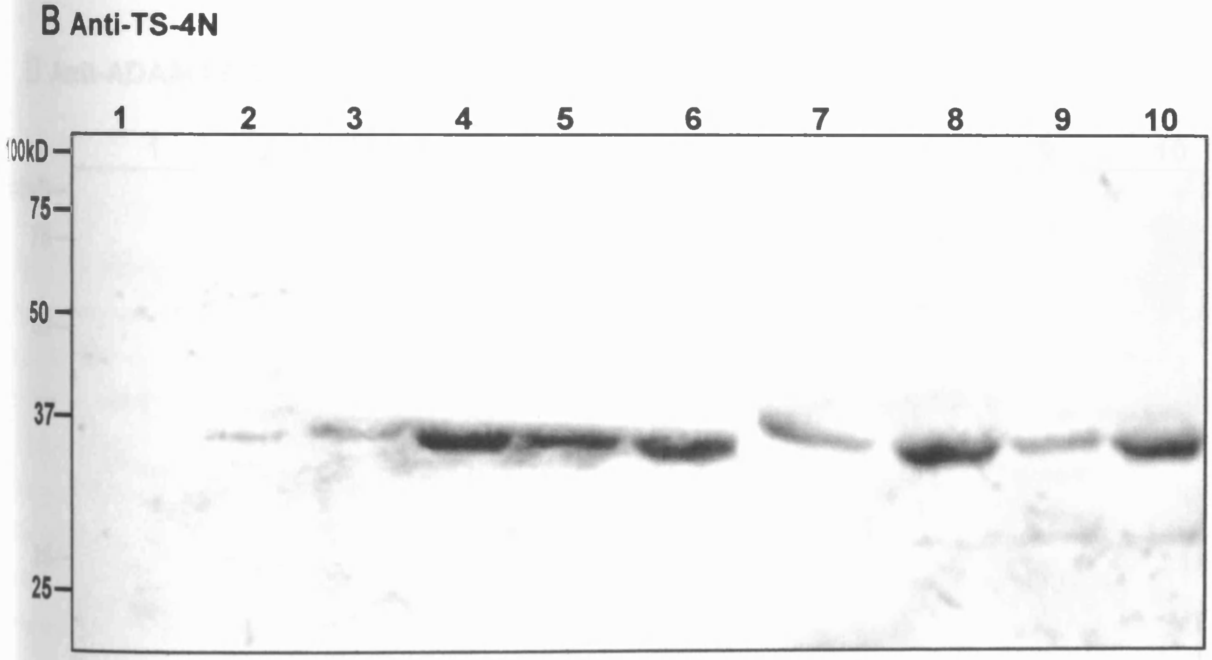
The polyclonal antibody to the prodomain of ADAMTS-5 detected low molecular weight zinc chelator bound bands, at 55 and 30kD (Figure 5.7D). The 55kD ADAMTS-5 isoform was detected in media from control and IL-1α treated cultures at all time points tested. In contrast, the 30kD isoform was detected in media samples from control cultures, but was absent from IL-1α treated cultures (Figure 5.7D). Both of these low molecular weight isoforms may result from enzyme catalysis.

- ## Heparin-Sepharose Bound Media Fractions

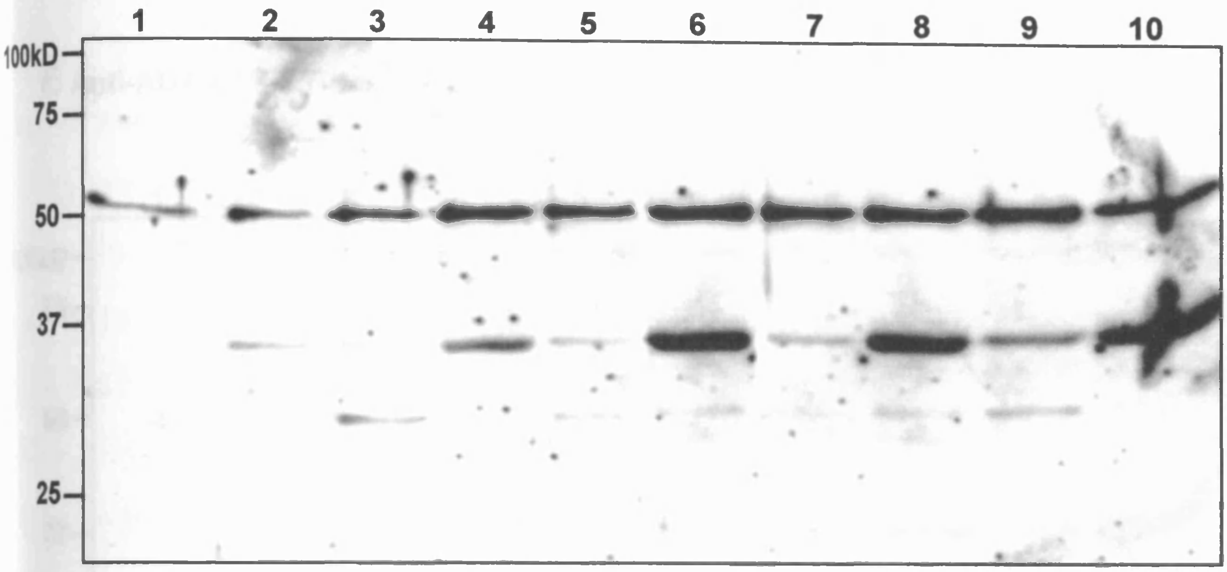
Media Samples Partially Purified via Passage over Heparin-Sepharose

Western blot analysis of the heparin bound fractions reveals a less complex pattern of immunopositive bands than those seen in zinc bound media fractions with antibodies recognising domains of ADAMTS-4 and -5, perhaps representing non-matrix associated populations of enzymes. Interestingly, the predominant heparin bound isoforms of ADAMTS-4 and –5 co-migrate at 37kD (Figures 5.8 A - E) with an additional 55kD isoform of ADAMTS-4 detected by the antibody to the spacer domain of the protein (Figure 5.8C). The heparin bound 37kD isoforms of ADAMTS-4 and –5 were detected in increased amounts in IL-1α treated cultures compared to controls and in addition, the intensity of the heparin bound 37kD isoforms of ADAMTS-4 and –5 also increased with increasing treatment time. These low molecular weight isoforms may result from enzyme catalysis during the catabolism of aggrecan. Alternatively, the co-migrating 37kD isoforms of ADAMTS-4 and –5 may result from alternatively spliced forms of the enzymes. The 37kD co-migrating isoforms detected by the antibodies to the prodomains of ADAMTS-4 and -5 will be inactive due to them possessing a prodomain (Figures 5.8 A and D). The 37kD co-migrating isoforms detected by the antibodies to the spacer domains of ADAMTS-4 and -5 are likely to be inactive, if they result from enzyme catalysis, as they are too small to contain both the metalloproteinase and spacer domains (Figures 5.8 C and E). The 37kD band detected by Anti-TS-4N is likely to contain a mixed population of ADAMTS-4 isoforms, including the inactive 37kD isoform which is also detected by the antibody to the prodomain of ADAMTS-4, and a Furin cleaved catalytically active form of the enzyme (Figure 5.8B).

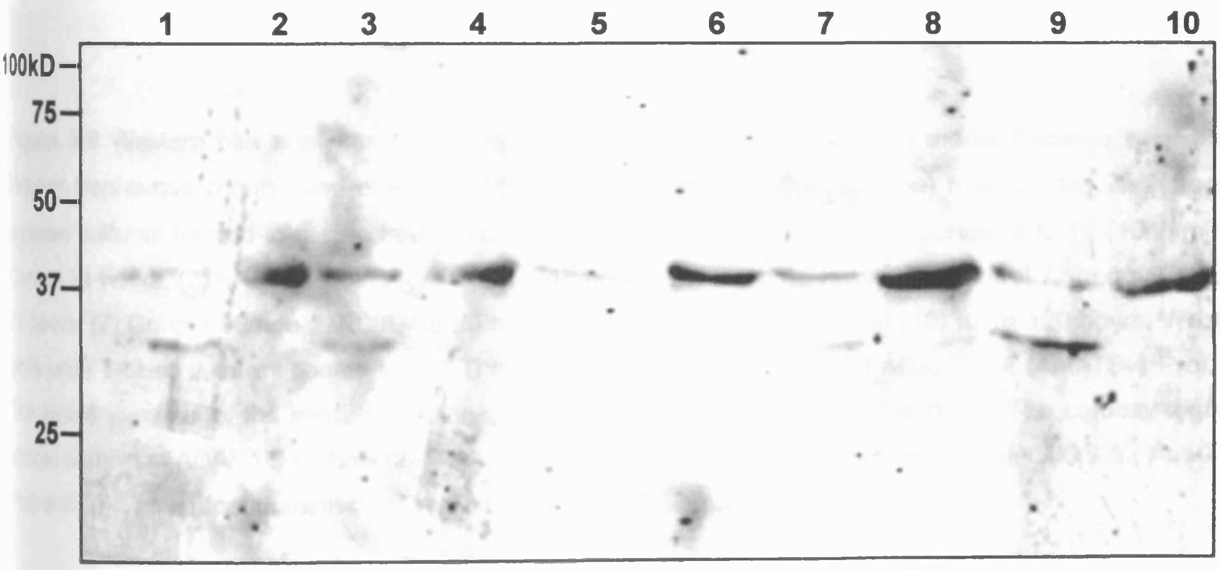

A Anti-ADAMTS-4 Pro
1 2 3 4 5 6 7 8 9 10
100kD
75
50
37
25


B Anti-TS-4N
1 2 3 4 5 6 7 8 9 10
100kD
75
50
37
25

C Anti-ADAMTS-4 Spacer



D Anti-ADAMTS-5 Pro



E Anti-ADAMTS-5 Spacer

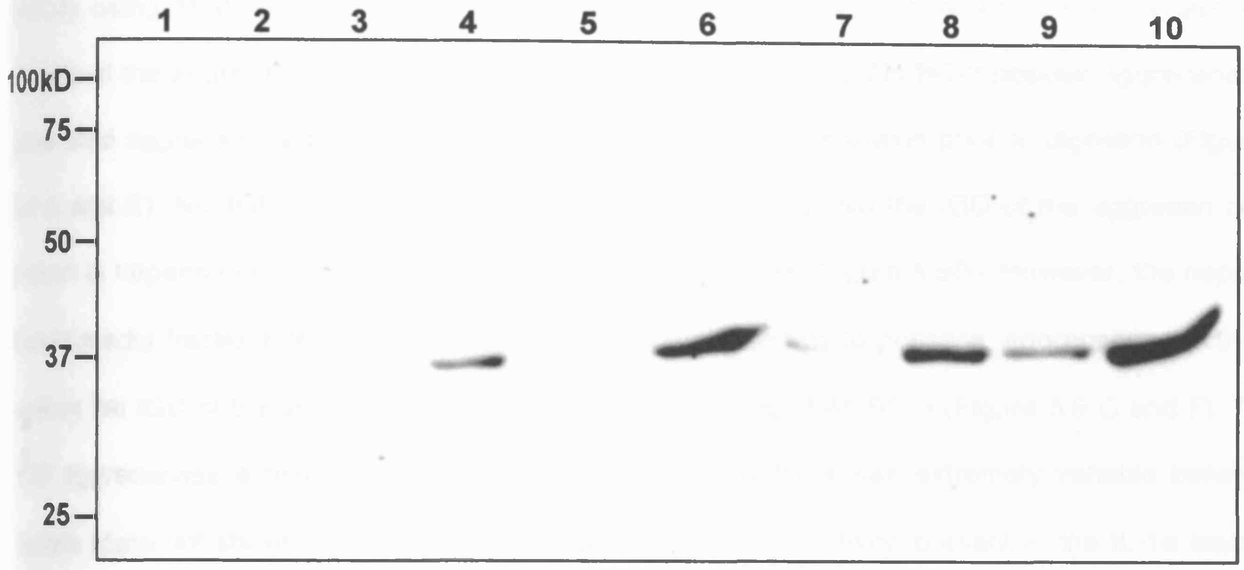


Figure 5.8 Western blot analyses of ADAMTS-4 and -5 isoforms present in media fractions bound to a Heparin-Sepharose column and eluted in 0.8M sodium chloride (50µl per lane) from 21 day chondrocyte-agarose cultures treated in serum free medium in the absence (control) or presence of IL-1α (10ng/ml) (1) Control 24 hours, (2) IL-1α 24 hours, (3) Control 48 hours, (4) IL-1α 48 hours, (5) Control 72 hours, (6) IL-1α 72 hours, (7) Control 96 hours, (8) IL-1α 96 hours, (9) Control 120 hours and (10) IL-1α 120 hours. Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (Anti-TS-4 Pro), (B) The amino-terminus of the metalloproteinase domain of ADAMTS-4 (Ant-TS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (Anti-TS-Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (Anti-TS-5 Pro) and (E) The spacer domain of ADAMTS-5 (Anti-TS-5 Spacer).

5.4.7 Presence of 'IGD Aggrecanase Activity' in Heparin and Zinc Chelator Bound Media Fractions using Exogenous A1D1 as a Substrate

The 'IGD aggrecanase activity' of the heparin and zinc chelator bound fractions of medium from cultures treated with and without IL-1α for 96 hours was assessed against purified aggrecan (A1D1) using M'Ab BC-3 to detect cleavage at the Glu^{373}-Ala^{374} bond within the interglobular domain of the aggrecan core protein (the 'IGD aggrecanase site'). No BC-3 positive aggrecanase-generated aggrecan fragments were detected in the A1D1 preparation prior to digestion (Figures 5.9 A and E). No 'IGD aggrecanase activity' was detected against the IGD of the aggrecan core protein in heparin bound media fractions from control cultures (Figure 5.9B). However, the heparin bound media fractions from IL-1α treated cultures were shown to possess 'aggrecanase activity' against the IGD of the aggrecan core protein detected using M'Ab BC-3 (Figure 5.9 C and F). The 'IGD aggrecanase activity' of the zinc chelator bound fractions was extremely variable between digests (data not shown). The intensity of 'IGD aggrecanase activity' present in the IL-1α treated heparin bound media fractions (i.e. that detected as BC-3 positive staining) was markedly reduced by preincubation with M'Ab Anti-TS-4N (Figure 5.9D), but was not significantly decreased by preincubation with a control M'Ab (70.6 Anti-Decorin) (Figure 5.9G). This result suggests that isoforms of ADAMTS-4 were responsible for the 'IGD aggrecanase activity' present in these heparin bound media fractions.

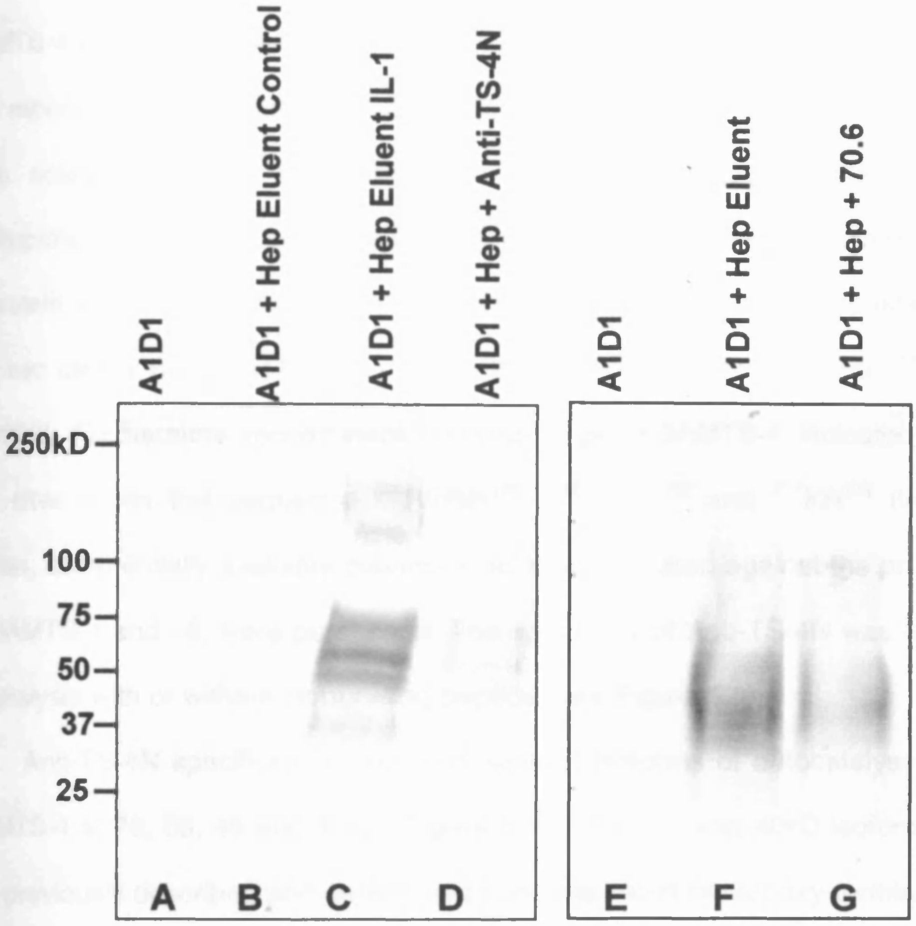


Figure 5.9 Western blot analysis of samples of purified aggrecan (A1D1) digested with heparin bound media fractions from cultures treated in the absence (control) and presence of IL-1α (10ng/ml) for 96 hours (A) and (E) Undigested aggrecan (A1D1), (B) A1D1 digested with heparin column eluent from control cultures, (C) and (F) A1D1 digested with heparin column eluent from IL-1α treated cultures, (D) A1D1 digested with heparin column eluent from IL-1α treated cultures following preincubation with M'Ab Anti-TS-4N and (G) A1D1 digested with heparin column eluent from IL-1α treated cultures following preincubation with control M'Ab (70.6 Anti-Decorin). Western blots were probed with M'Ab BC-3 detecting the aggrecanase-generated interglobular domain neoepitope ^{374}ARGSV... Samples were deglycosylated prior to electrophoretic separation.

5.5 Discussion

In the presence of IL-1α the aggrecan present in the matrix secreted by chondrocytes embedded in agarose is degraded by aggrecanases, two of which have been identified as ADAMTS-4 and –5. In order to investigate the secretion, sequestration and activation of ADAMTS-4 we report a new monoclonal antibody (M'Ab) Anti-TS-4N, which has been generated against amino acids within the sequence ^{213}FASLSRFV^{220} located at the amino-terminus of the metalloproteinase domain of ADAMTS-4. This sequence is thought to form the amino-terminus of the protein in Furin cleaved ADAMTS-4 (Molloy *et al.*, 1992, and Tortorella *et al.*, 1999). Purified Furin can cleave pro-ADAMTS-4 *in vitro* within the consensus sequence ^{206}RPRRAKR^{212} (Gao *et al.*, 2002). Furthermore recombinant mutants of pro-ADAMTS-4 indicated cleavage to occur at three sites within this sequence ^{206}RPRR^{209}, ^{209}RAKR^{212} and ^{211}KR^{212} (Wang *et al.*, 2004). In addition, commercially available polyclonal antibodies raised against the pro- and spacer domains of ADAMTS-4 and –5, were purchased. The specificity of Anti-TS-4N was determined by Western blot analysis with or without immunising peptide (see Figure 5.3).

Anti-TS-4N specifically recognised several isoforms of autocatalysed recombinant human ADAMTS-4 at 75, 55, 45 and 40kD (Figure 5.3A). The 55 and 40kD isoforms of ADAMTS-4 have been previously described and determined to be the result of carboxy-terminal truncation (Flannery *et al.*, 2002). However, the polyclonal antibody to the carboxy-terminal spacer domain also recognised these isoforms of ADAMTS-4 (Figure 5.4C). This suggests the presence of mixed populations of co-migrating isoforms of ADAMTS-4 where some isoforms have undergone carboxy-terminal truncation, as described by Flannery *et al.*, 2002, and others amino-terminal truncation, resulting in isoforms of similar molecular weights.

Both of the antibodies raised against ADAMTS-5 recognised a 90kD isoform of the recombinant protein (Figures 5.4 D and E) which may represent the intact zymogen form of the enzyme (predicted molecular weight 101.7kD). The isolated prodomain of ADAMTS-5, along with other carboxy-terminally truncated isoforms of the enzyme, were detected by the polyclonal antibody to the prodomain as a series of small bands between 15-30kD (Figure 5.4D). As expected the small isoforms were not detected by the polyclonal antibody to the spacer domain of ADAMTS-

5 (Figure 5.4E). Interestingly, the antibody to the spacer domain of ADAMTS-5 did not detect an isoform corresponding to the Furin cleaved form (predicted molecular weight 73.6kD) (Figure 5.4E). However, silver staining for total protein present in the preparation of human recombinant ADAMTS-5 detected bands at 105, 75 and 45kD as well as between 20-35kD (Figure 5.4F). The 75kD isoform detected is predicted to be the Furin cleaved isoform of the enzyme (predicted molecular weight 73.6kD). Interestingly, the 45kD isoform was also not detected by the antibody to the spacer domain of ADAMTS-5 which may be due to masking of the epitope (Figure 5.4E).

The molecular weight of the ADAMTS-4 and -5 isoforms detected in media samples and detergent extracts of agarose plugs from chondrocyte-agarose cultures differ from those described in Section 5.4.3 for the recombinant enzymes. This may be due to species variation as the predicted molecular weights and recombinant protein preparations used were human and the chondrocyte-agarose cultures used porcine articular chondrocytes.

Isoforms of ADAMTS-4 and -5 detected in detergent extracts of agarose plugs may be sequestered in the extracellular matrix, membrane-bound or associated, or located intracellularly. Western blots, using antibodies to ADAMTS-4 and -5, of detergent extracts from control and IL-1α treated cultures, as well as a time zero matrix, all showed isoforms of ADAMTS-4 and -5 with apparent equal staining intensity (Figures 5.5 and 5.6). A 100kD isoform of ADAMTS-4 was detected in detergent extracts of agarose plugs by all of the antibodies raised against sequences in different domains of the protein (Figures 5.5 A-C and 5.6 A-C) and could represent the zymogen form of the enzyme (predicted human molecular weight 90.2kD) (Figure 5.10A). A 110kD isoform of ADAMTS-4 was described by Pratta *et al.*, which was detected, using a polyclonal antibody to the peptide sequence ^{394}VMAH^{397} within the metalloproteinase domain of the enzyme, in cell lysates from bovine monolayers and predicted to correspond to the intact zymogen form of ADAMTS-4 (Pratta *et al.*, 2003). All three antibodies raised against ADAMTS-4 also detected bands at 75 and 55kD as well as a broad band at ~60kD (Figures 5.5 A-C and 5.6 A-C). The 75 and 60kD bands detected may represent the intact Furin processed active form of ADAMTS-4 (predicted human molecular weight 67.9kD) (Figure 5.10C). However, since these bands were also detected by the polyclonal antibody to the amino-terminal prodomain of the enzyme (Figures 5.5A and 5.6A) this may indicate a truncated protein containing the prodomain associated with other matrix molecules (Figure 5.10B). A 55kD isoform of ADAMTS-4 was described by Pratta *et al.*, which was detected, using a polyclonal antibody to the peptide sequence ^{394}VMAH^{397} within the metalloproteinase domain of the enzyme, in cell lysates from bovine monolayers (Pratta *et al.*, 2003) and was predicted to result from carboxy-terminal truncation (Flannery *et al.*, 2002, and Gao *et al.*, 2004). However, since the 55kD isoform was detected by the antibody to the spacer domain of ADAMTS-4 (Figures 5.5C and 5.6C) this may indicate the presence of isoforms of the enzyme which have either undergone carboxy- or amino-terminal truncation resulting in isoforms of similar size (Figures 5.10 E and D, respectively). In conclusion staining of these bands with all three antibodies suggests the presence of mixed populations of co-migrating isoforms of the enzyme.

A 100kD isoform of ADAMTS-5 was detected by the antibody to the prodomain of the protein in detergent extracts of agarose plugs as well as in zinc chelator bound samples of media from control and IL-1α treated cultures (Figures 5.5D, 5.6D and 5.7D). This 100kD ADAMTS-5 isoform may be the zymogen form of the enzyme (predicted human molecular weight 101.7kD) (Figure 5.11A), however as no staining was seen with the antibody to the spacer domain (Figures 5.5E, 5.6E and 5.7E) it is more likely to be a smaller truncated form complexed with other matrix components such as fibronectin (Hashimoto *et al.*, 2004) (Figure 5.11B).

The polyclonal antibodies raised against sequences in ADAMTS-5 both detect bands at 75 and 60kD in detergent extracts of agarose plugs as well as in zinc chelator bound samples of media from control and IL-1α treated cultures (Figures 5.5 D and E, 5.6 D and E, and 5.7 D and E). Either, or both, of the isoforms detected by the antibody to the spacer domain of ADAMTS-5 may correspond to the Furin processed active form of the enzyme (predicted human molecular weight 73.6kD) (Figure 5.11C). The antibody to the spacer domain of ADAMTS-5 also detected a small isoform (32kD) of the enzyme in detergent extracts of agarose plugs from cultures treated with IL-1α which was absent from control cultures (Figure 5.6E), indicating catalysis of ADAMTS-5 to occur following treatment with IL-1α in serum free conditions.

Predominantly, high molecular weight isoforms of ADAMTS-4 and -5 failed to bind to the Heparin-Sepharose column and were bound by the Zinc Chelator column, whereas lower molecular weight isoforms were bound by the Heparin-Sepharose column. Interestingly, many of the higher molecular weight zinc bound isoforms of the enzymes, which failed to bind to the heparin column, posses the thrombospondin type I repeats, cysteine-rich and / or spacer domains thought to mediate GAG (and therefore heparin) binding. This suggests some form of structural interference preventing the GAG binding regions of ADAMTS-4 and -5 from interacting with the Heparin-Sepharose column. This structural interference is absent from the smaller molecular weight ADAMTS-4 and -5 isoforms allowing them to bind to the heparin column.

The zinc chelator bound isoforms of ADAMTS-4 and –5 detected showed no differences between the time points tested (Figure 5.7). All of the antibodies to ADAMTS-4 detected a predominant ~70kD isoform in media from control and IL-1α treated cultures (Figures 5.7 A-C), which could be the Furin-activated form of the enzyme (predicted human molecular weight 67.9kD) (Figure 5.10C). The presence of immunopositive bands of the same molecular weight detected by the antibody to the prodomain suggests the presence of mixed populations co-migrating active and inactive isoforms of ADAMTS-4.

Both antibodies to the pro- and metalloproteinase domains of ADAMTS-4 detected a zinc chelator bound band of 55kD in medium from control and IL-1α treated cultures (Figures 5.7 A and B). Flannery *et al.*, 2002 described a 55kD isoform of ADAMTS-4 which resulted from autocatalytic truncation of the enzyme through loss of the carboxy-terminal spacer domain, which may explain the lack of reactivity of the 55kD isoform of ADAMTS-4 with the polyclonal antibody raised against the spacer domain of the enzyme (Figure 5.7C). An alternative strategy for production of the 55kD isoform of ADAMTS-4 has recently been described by Gao *et al.*, (Gao *et al.*, 2004). GPI-anchored MT4-MMP binds to Furin-activated ADAMTS-4 (human predicted molecular weight 67.9kD) and cleaves at the Lys^{694}-Phe^{695} bond within the cysteine-rich domain resulting in a ~55kD isoform (Figure 5.10E) with an increased ability to cleave aggrecan at the 'aggrecanase site' compared to the 67.9kD isoform (Gao *et al.*, 2004).

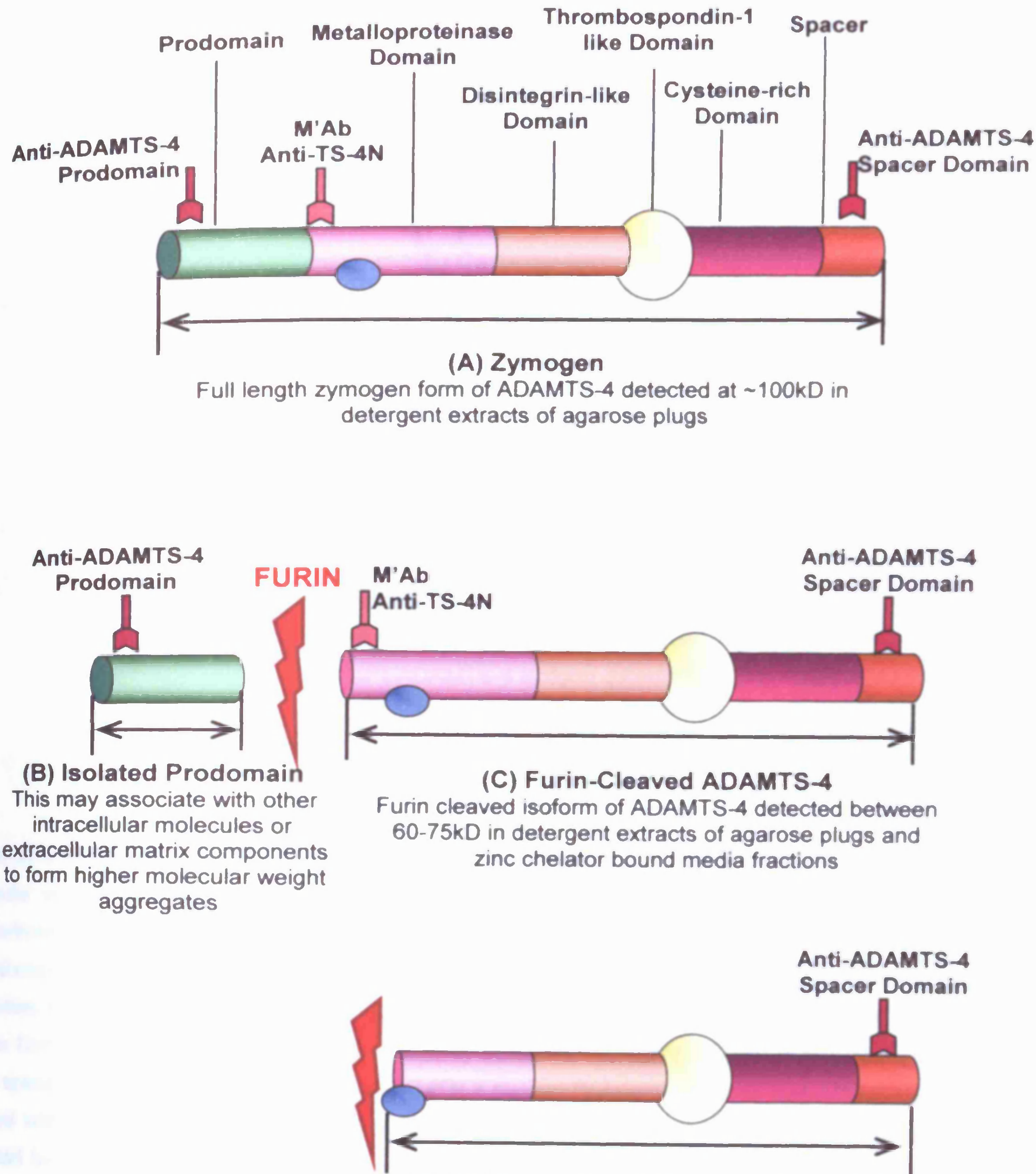
The predominant ~70kD zinc chelator bound isoform of ADAMTS-5 was detected by both the antibodies to the pro- and spacer domains of the enzyme in media samples from control and IL-1α treated cultures (Figures 5.7 D and E). Immunostaining detected by the antibody to the spacer domain of ADAMTS-5 suggests that a proportion of this band may represent furin activated enzyme (predicted human molecular weight 73.6kD) (Figure 5.11C).

The polyclonal antibody to the prodomain of ADAMTS-5 detected low molecular weight zinc chelator bound bands, at 55 and 30kD (Figure 5.7D). Both of these low molecular weight isoforms may result from enzyme catalysis (Figure 5.11G).

The predominant heparin bound isoforms of ADAMTS-4 and –5 all co-migrated at 37kD (Figures 5.8 A-E) with an additional 55kD isoform of ADAMTS-4 being detected by the antibody to the spacer domain of the protein (Figure 5.8C). The 55kD isoform is likely to result from amino-terminal truncation of ADAMTS-4 (Figure 5.10D). In detergent extracts of agarose plugs a 55kD immunopositive band was detected by all three antibodies to ADAMTS-4 (Figures 5.6 A-C and 5.7 A-C) indicating mixed populations of co-migrating isoforms some of which had undergone amino-terminal truncation and others carboxy-terminal truncation (Figures 5.10 D and E, respectively) as described by (Flannery *et al.*, 2002, and Gao *et al.*, 2004). In zinc chelator bound media fractions 55kD isoforms were detected by antibodies to the pro- and metalloproteinase domains of ADAMTS-4 (Figures 5.8 A and B). This indicates the mixed population of co-migrating 55kD isoforms of ADAMTS-4 detected in detergent extracts of agarose plugs to also be present in the experimental culture medium. These were partially separated by Heparin-Sepharose with amino-terminally truncated isoforms (Figure 5.10D) binding to the heparin and the carboxy-terminally truncated isoforms (Figure 5.10E) binding to the zinc chelator column. This may be due to the ability of the carboxy-terminal regions of ADAMTS-4 to interact with sulphated GAGs including heparin (Flannery *et al.*, 2002).

The intensity of immunopositive staining of the heparin bound 37kD isoforms of ADAMTS-4 and –5 increases significantly over the treatment time and in IL-1α treated cultures compared to controls (Figures 5.8A-E). This is similar to previously published results indicating treatment with IL-1β to increase the prevalence of lower molecular weight isoforms of ADAMTS-4 in cell lysates from bovine chondrocyte monolayers (Pratta *et al.*, 2003). The co-migrating 37kD isoforms of ADAMTS-4 and -5 may result from enzyme catalysis either by autocatalysis due to a lack of suitable substrate or activation of soluble forms of the enzymes. Agarose cultures treated in the presence of IL-1α release over 70% of the sulphated GAG present to the medium in 24 hours (see Chapter 4). Therefore following 24 hours of treatment in the presence of IL-1α 70% of the substrate for ADAMTS-4 and –5 has been lost to the medium, consequently by 48 hours of treatment in the presence of IL-1α the enzymes themselves may be undergoing autocatalysis. Then again, a model for generation of 55 and 40kD isoforms of ADAMTS-4 was recently described in which catalysis occurs at the cell surface via membrane bound MT4-MMP (Gao *et al.*, 2004). In this model cleavage occurs at the Lys^{694}-Phe^{695} bond within the cysteine-rich domain resulting in a ~55kD isoform and at the Thr^{581}-Phe^{582} bond within the thrombospondin-1 like domain resulting in a ~40kD isoform. The 40kD isoform described using human peptide sequence by Gao *et al.*, 2004 may have a molecular weight of ~37kD in porcine chondrocytes (Figure 5.10 F and G). A similar mechanism of cleavage may be proposed for ADAMTS-5 (Figure 5.11G and H). Alternatively, the 37kD isoforms of ADAMTS-4 and –5 may result from alternative splicing or proteolytic activation of the enzyme. Since these 37kD isoforms of ADAMTS-4 and –5 are increased in IL-1α treated cultures compared to controls they may play a role in the increased 'IGD aggrecanase activity' detected in these cultures (see Chapter 4).

The heparin bound media fractions from cultures treated with IL-1α for 96 hours possess activity against the Glu^{373}-Ala^{374} bond ('aggrecanase site') within the interglobular domain of the core protein of purified aggrecan (A1D1) (Figures 5.9 C and F). This 'IGD aggrecanase activity' was decreased by preincubation with monoclonal antibody Anti-TS-4N. This indicates the 'IGD aggrecanase activity' of the heparin bound media fractions to be mainly due to ADAMTS-4 isoforms containing the sequence ^{213}FASLSRFV^{220}. Therefore a proportion of the 37kD heparin bound isoforms of ADAMTS-4 detected by Anti-TS-4N possess 'IGD aggrecanase activity'. Previously published data indicated ADAMTS-4 to be the key player in IL-1 induced 'IGD aggrecanase activity'. Immunodepletion of ADAMTS-4 from the medium of IL-1 stimulated bovine articular cartilage explants led to a 75% reduction in 'IGD aggrecanase activity' against purified exogenous aggrecan as detected using a BC-3 ELISA. In contrast, immunodepletion with an ADAMTS-5 antibody decreased the detected 'IGD aggrecanase activity' by only 15% (Tortorella *et al.*, 2001). Furthermore it has recently been reported that carboxy-terminal truncation enhances the 'IGD aggrecanase activity' of ADAMTS-4 (Gao *et al.*, 2002, and Kashiwagi *et al.*, 2004), thus implying a role for the carboxy-terminally truncated 37kD isoforms of ADAMTS-4 and, possibly –5, in the increased 'IGD aggrecanase activity' seen in the presence of IL-1α (Chapter 4).

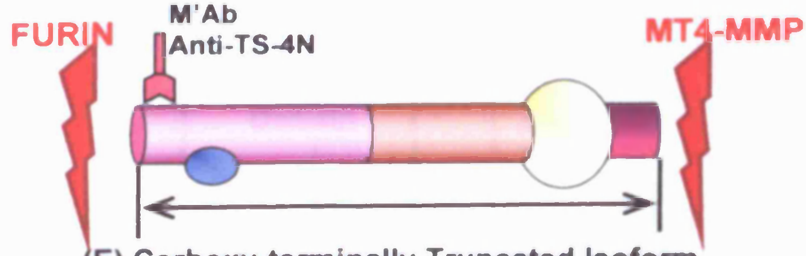


(A) Zymogen
Full length zymogen form of ADAMTS-4 detected at ~100kD in detergent extracts of agarose plugs

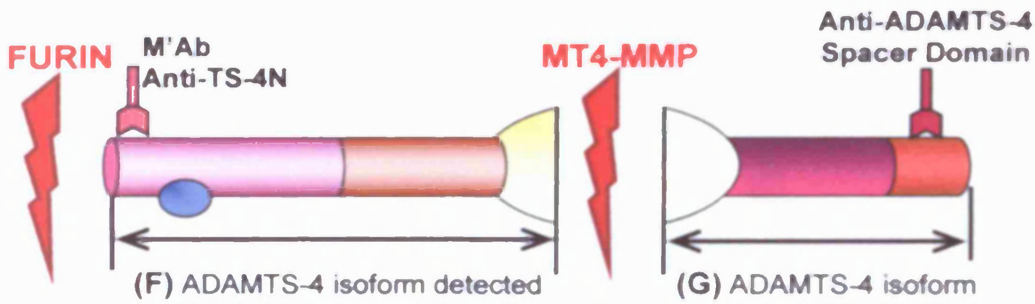
(B) Isolated Prodomain
This may associate with other intracellular molecules or extracellular matrix components to form higher molecular weight aggregates

(C) Furin-Cleaved ADAMTS-4
Furin cleaved isoform of ADAMTS-4 detected between 60-75kD in detergent extracts of agarose plugs and zinc chelator bound media fractions

(D) Amino-terminally Truncated Isoform
ADAMTS-4 isoform detected ~50-55kD in detergent extracts of agarose plugs and heparin bound media fractions



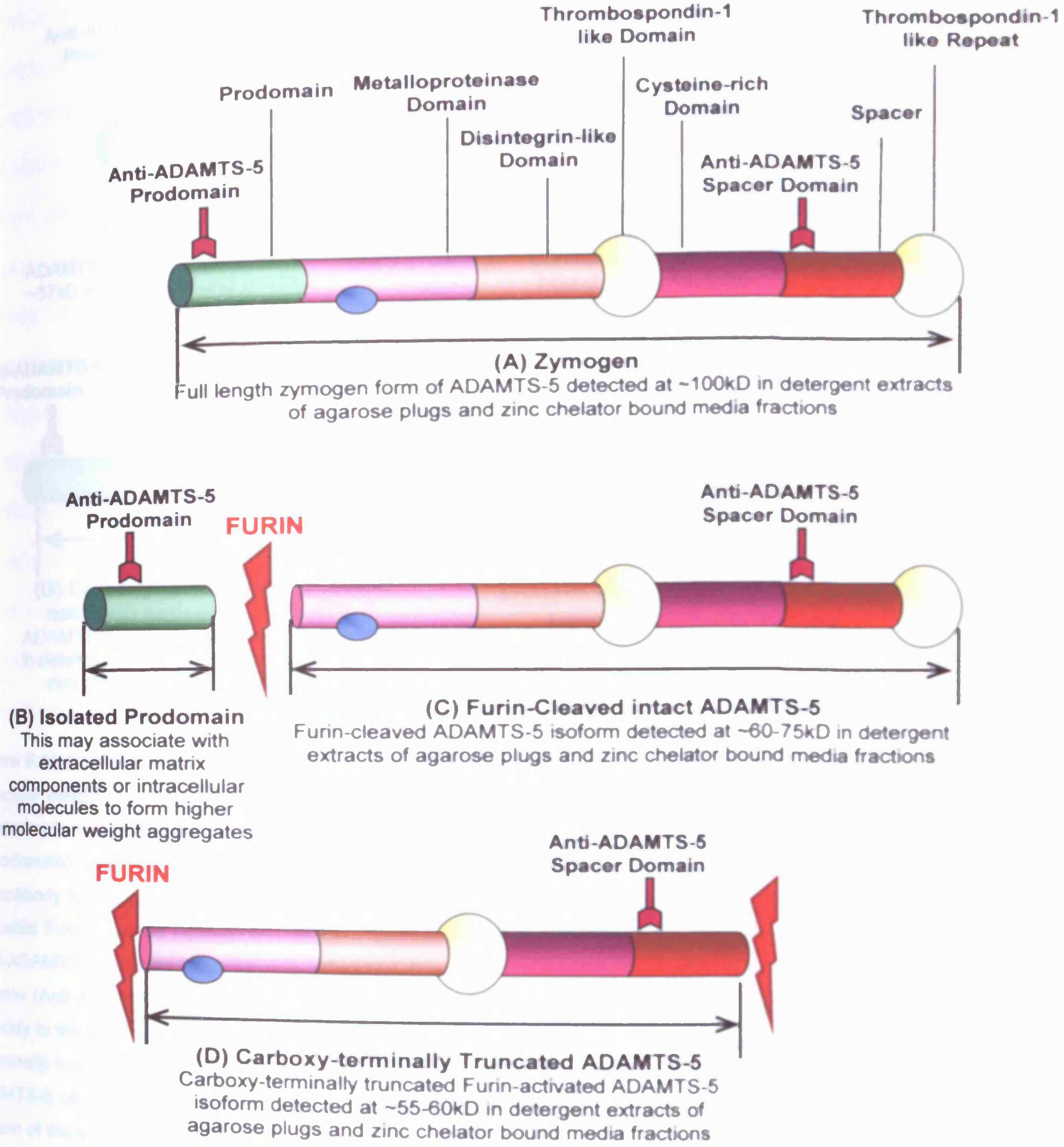
(E) Carboxy-terminally Truncated Isoform
ADAMTS-4 isoform detected ~50kD in detergent extracts of agarose plugs and zinc chelator bound media samples



(F) ADAMTS-4 isoform detected ~37kD in heparin bound media fractions from IL-1 treated cultures

(G) ADAMTS-4 isoform detected ~37kD in heparin bound media fractions

Figure 5.10 Potential ADAMTS-4 isoforms. (A) Full length zymogen form of ADAMTS-4 (human predicted molecular weight 90.2kD) detected by all antibodies to ADAMTS-4. (B) Isolated prodomain of ADAMTS-4 (approximate molecular weight ~16kD) detected by antibody to the prodomain of ADAMTS-4 (Anti-ADAMTS-4 Prodomain). (C) Furin-activated intact ADAMTS-4 (human predicted molecular weight 67.9kD) detected by antibodies to the metalloproteinase and spacer domains of ADAMTS-4 (Anti-TS-4N and Anti-ADAMTS-4 Spacer Domain, respectively). (D) Amino-terminally truncated ADAMTS-4 isoform detected by the antibody to the spacer domain of ADAMTS-4 (Anti-ADAMTS-4 Spacer Domain). (E) Furin cleaved ADAMTS-4 isoform cleaved within the cysteine-rich domain by MT4-MMP at the Lys^{694}-Phe^{695} bond (Gao *et al.*, 2004) and detected by the antibody to the metalloproteinase domain of ADAMTS-4 (Anti-TS-4N). (F) Furin cleaved ADAMTS-4 isoform cleaved within the thrombospondin-1 like domain by MT4-MMP at the Thr^{581}-Phe^{582} bond (Gao *et al.*, 2004) and detected by the antibody to the metalloproteinase domain (Anti-TS-4N. (G) ADAMTS-4 isoform resulting from cleavage by MT4-MMP at the Thr^{581}-Phe^{582} site within the thrombospondin-1 type domain and detected by the antibody to the spacer domain of ADAMTS-4 (Anti-ADAMTS-4 Spacer domain).


Thrombospondin-1
like Domain
Thrombospondin-1
like Repeat
Prodomain
Metalloproteinase
Domain
Cysteine-rich
Domain
Spacer
Disintegrin-like
Domain
Anti-ADAMTS-5
Prodomain
Anti-ADAMTS-5
Spacer Domain
(A) Zymogen
Full length zymogen form of ADAMTS-5 detected at ~100kD in detergent extracts of agarose plugs and zinc chelator bound media fractions
Anti-ADAMTS-5
Prodomain
FURIN
Anti-ADAMTS-5
Spacer Domain
(B) Isolated Prodomain
This may associate with extracellular matrix components or intracellular molecules to form higher molecular weight aggregates
(C) Furin-Cleaved intact ADAMTS-5
Furin-cleaved ADAMTS-5 isoform detected at ~60-75kD in detergent extracts of agarose plugs and zinc chelator bound media fractions
Anti-ADAMTS-5
Spacer Domain
FURIN
(D) Carboxy-terminally Truncated ADAMTS-5
Carboxy-terminally truncated Furin-activated ADAMTS-5 isoform detected at ~55-60kD in detergent extracts of agarose plugs and zinc chelator bound media fractions

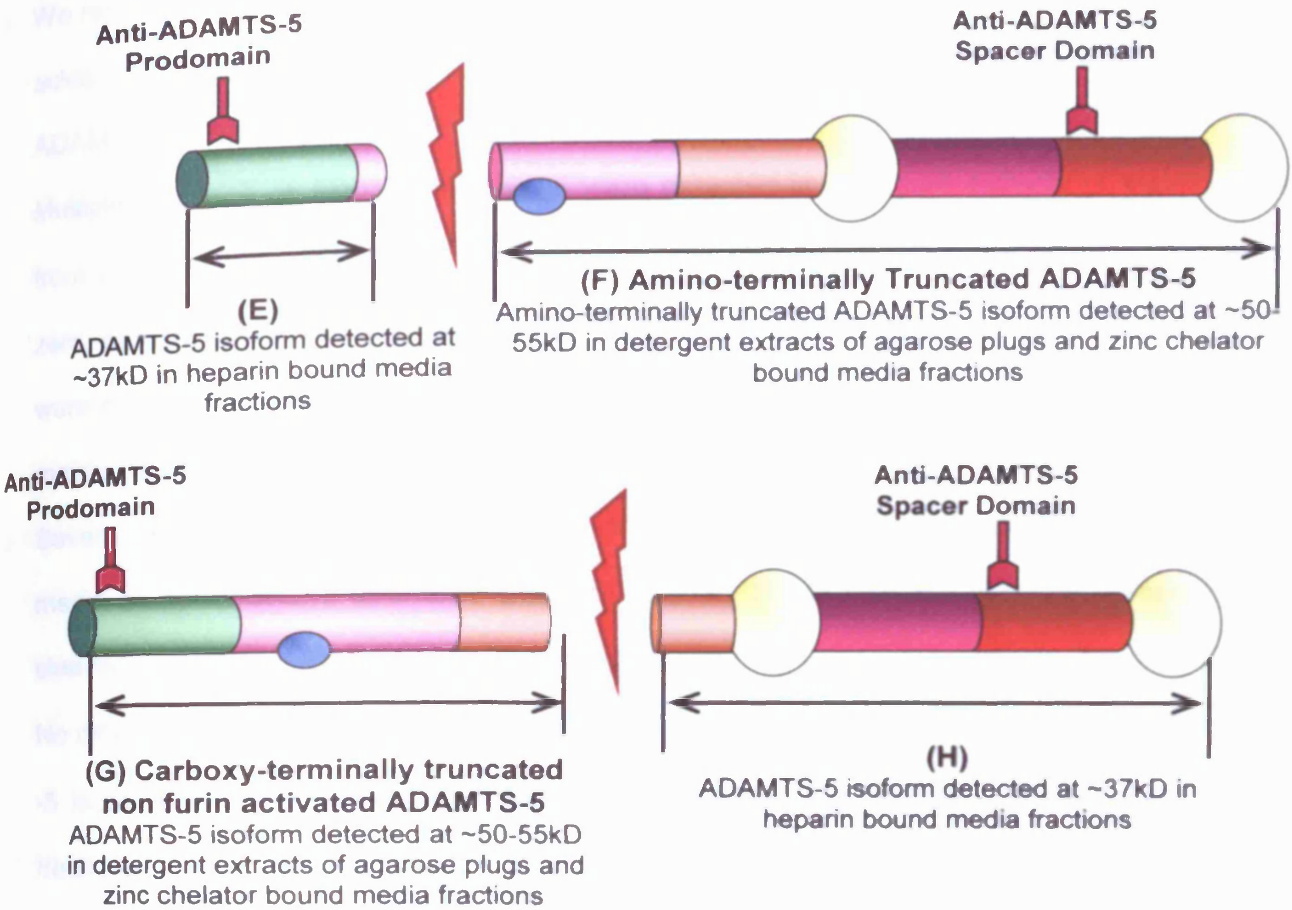


Figure 5.11 Potential ADAMTS-5 isoforms. (A) Full length zymogen form of ADAMTS-5 (human predicted molecular weight 101.7kD) detected by both antibodies to ADAMTS-5. (B) Isolated prodomain of ADAMTS-5 (approximate molecular weight ~25kD) detected by antibody to the prodomain of ADAMTS-5 (Anti-ADAMTS-5 Prodomain). (C) Furin cleaved intact ADAMTS-5 (human predicted molecular weight 73.6kD) detected by the antibody to the spacer domain of ADAMTS-5 (Anti-ADAMTS-5 Spacer Domain). (D) Carboxy-terminally truncated Furin cleaved ADAMTS-5 isoform detected by the antibody to the spacer domain of ADAMTS-5 (Anti-ADAMTS-5 Spacer Domain). (E) ADAMTS-5 isoform detected by the antibody to the prodomain of the enzyme (Anti-ADAMTS-5 Prodomain). (F) Amino-terminally truncated ADAMTS-5 isoform detected by the antibody to the antibody to the spacer domain of the enzyme (Anti-ADAMTS-5 Spacer domain). (G) Carboxy -terminally truncated non Furin cleaved ADAMTS-5 isoform detected by the antibody to the prodomain of ADAMTS-5 (Anti-ADAMTS-5 Prodomain). (H) ADAMTS-5 isoform detected by the antibody to the spacer domain of the enzyme (Anti-ADAMTS-5 Spacer domain).

5.6 Summary

- We report the characterisation of a new M'Ab Anti-TS-4N which specifically recognises amino acids within the sequence ^{213}FASLSRFV^{220} located at the amino-terminus of Furin cleaved ADAMTS-4.
- Multiple isoforms of ADAMTS-4 and -5 were detected in detergent extracts of agarose plugs from chondrocyte-agarose cultures treated in the absence or presence of IL-1α and at time zero, prior to treatment in serum free conditions, which may represent enzyme isoforms which were membrane bound / associated, located intracellularly or sequestered in the extracellular matrix. These included potential zymogen, Furin cleaved and truncated enzyme isoforms.
- Several high molecular weight isoforms of ADAMTS-4 and -5 were detected in fractions of media samples bound to a Zinc Chelator column. These included potential zymogen, Furin cleaved and truncated enzyme isoforms.
- No differences were detected between control and IL-1α treated cultures in the ADAMTS-4 and -5 isoforms present in detergent extracts of agarose plugs and zinc chelator bound media fractions.
- In media fractions bound by a Heparin-Sepharose column a series of low molecular weight co-migrating 37kD isoforms of ADAMTS-4 and -5 were detected in apparently increased amounts with increasing treatment time and in IL-1α treated cultures compared to controls.
- Heparin bound media fractions from IL-1α treated cultures, but not controls, possess 'IGD aggrecanase activity' against exogenous purified aggrecan.
- The 'IGD aggrecanase activity' of heparin bound media fractions from IL-1α treated cultures was ablated by addition of M'Ab Anti-TS-4N. Therefore indicating the 'IGD aggrecanase activity' of heparin bound media fractions to reside in a low molecular weight, 37kD, isoform of ADAMTS-4.

Chapter 6: Analysis of Tissue Inhibitor of MetalloProteinase-3 (TIMP-3)

6.1 Introduction

The family of Tissue Inhibitors of MetalloProteinases (TIMPS) currently comprises 4 members in humans: TIMP-1, -2, -3 and -4. These are homologous in sequence and have similar secondary and tertiary structures. Mammalian TIMPs are two-domain molecules, having amino-terminal domains of ~125 amino acids and smaller carboxy-terminal domains of ~65 residues. Each domain is stabilised by three disulphide bonds (Williamson *et al.*, 1990).

TIMP-3 has several properties distinct from those of other TIMPs, which include its ability to bind tightly to the extracellular matrix via interaction of its amino-terminal domain with polyanionic components of the extracellular matrix (Pavloff *et al.*, 1992, and Yu *et al.*, 2000). The amino-terminal domain of TIMP-3 has been shown to inhibit the catalytic activity of both ADAMTS-4 and –5 (Kashiwagi *et al.*, 2001). This interaction is not unique to TIMP-3 as TIMPs-1, -2 and -4 also bind to ADAMTS-4, but with much lower affinity than TIMP-3 (Hashimoto *et al.*, 2001). The amino-terminal portion of TIMP-3 has been proposed to interact with ADAMTS-4 and –5 via their metalloproteinase domains and is postulated to be their natural inhibitor.

ADAMTS-4 and -5 isoforms were detected sequestered in the matrix in chondrocyte agarose cultures (Chapter 5 Sections 5.4.4 and 5.4.5), as well as released to the culture medium (Section 5.4.6), in the absence (control) and presence of IL-1α. Many of these isoforms were detected in control cultures and sequestered in the agarose plugs prior to treatment in serum free conditions. In order to determine whether these isoforms of ADAMTS-4 and -5 were inactive or were being inactivated by the presence of their proposed natural inhibitor, TIMP-3, a polyclonal antibody raised against the carboxy-terminal domain of TIMP-3 (RP2T3) was purchased.

Kashiwagi *et al.*, have recently developed a purification method for ADAMTS-4 and -5 using a recombinant protein comprising the amino-terminal portion of TIMP-3 (N-TIMP-3) to bind the enzymes allowing them to be co-purified by binding of the Tag on the N-TIMP-3 to a nickel agarose column (Kashiwagi *et al.*, 2004). This purification system was used to examine the isoforms of ADAMTS-4 and -5 present in media samples and detergent extracts of agarose plugs from chondrocyte-agarose cultures which were able to bind the amino-terminal domain of TIMP-3.

These isoforms of ADAMTS-4 and -5 were analysed using the mono- and polyclonal antibodies to domains of ADAMTS-4 and -5 described in Chapter 5 (illustrated in Figure 5.1).

In addition, the 'IGD aggrecanase activity' present in the medium of chondrocyte-agarose cultures was analysed against purified porcine aggrecan (A1D1) in the presence and absence of full length human recombinant TIMP-3 and the recombinant protein comprising the amino-terminal portion of human TIMP-3 (N-TIMP-3).

6.2 Materials

- *Polyclonal antibody RP2T3 raised against the carboxy terminal region of TIMP-3 was obtained from Triple Point Biologics Inc., Forest Grove, OR, US.*
- *Human recombinant TIMP-3 was obtained from Triple Point Biologics Inc., Forest Grove, OR, US.*
- *Alkaline Phosphatase linked goat anti-rabbit secondary antibody was obtained from Sigma-Aldrich, Poole, Dorset, UK.*
- *N-TIMP-3 was a kind gift from Dr. Masahide Kashiwagi and Professor Hideaki Nagase, Imperial College, The Kennedy Institute of Rheumatology, London, UK.*
- *Nickel-Agarose was obtained from Sigma-Aldrich, Poole, Dorset, UK.*
- *All other reagents were of laboratory grade and are listed in Chapter 2 Section 2.1 and Chapter 4 Section 4.2.*

6.3 Methods

6.3.1 Western blot analysis of TIMP-3

Experimental media samples or detergent extracts of agarose plugs (prepared in Chapter 4 Section 4.3.1 and Chapter 2 Sections 2.2.9 and 2.2.10) (all 50µl per lane) and samples of recombinant human TIMP-3 (0.5µg protein per lane) were prepared in Laemmli sample buffer (Laemmli 1970) with (reducing conditions) or without (non-reducing conditions) 10% (v/v) β-mercaptoethanol, and electrophoresed on 12% SDS-PAGE slab gels in running buffer. The gels were then transferred onto Nitrocellulose membrane (0.22µ) in transfer buffer at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis. Membranes were blocked in 5% (w/v) Bovine Serum Albumen (BSA) in Tris Saline Azide (TSA) for a minimum of 1 hour at room temperature with rocking. The membranes were washed 3 x 10 minutes in Phosphate Buffered Saline with 0.1% Tween 20 (PBS-T) and incubated with antibody RP2T3 (a polyclonal antibody to the carboxy terminal region of TIMP-3) diluted 1:1000 in 1% (w/v) BSA in TSA overnight at room temperature with rocking. The blots were washed 3 x 10 minutes in PBS-T and incubated in alkaline phosphatase linked goat anti-rabbit secondary antibody diluted 1:1000 in 1% (w/v) BSA in TSA for 1 hour at room temperature with rocking. The membranes were washed 3 x 10 minutes in PBS-T and developed in Nitro Blue Tetrazolium (NBT-50mg/ml in dimethylformamide) and 5-Bromo-4-Chloro-3-Indoyl Phosphate (BCIP-50mg/ml in dimethylamide), 66µl NBT and 33µl BCIP per 10ml Alkaline Phosphatase (AP) buffer (100mM tris, 100mM sodium chloride, 5mM magnesium chloride pH 9.55) (adapted from Hughes *et al.*, 1998).

6.3.2 Purification of ADAMTS-4 and -5 Isoforms using N-TIMP-3 and a Nickel-Agarose Column

Aliquots of media and detergent extracts of agarose plugs (500µl), from cultures treated in the absence (control) or presence of IL-1α for 96 hours, were added to 100nM human recombinant N-TIMP-3 and mixed. The mixture was then dialysed against a 100x volume of wash buffer (50mM tris HCl pH 7.5 with 100mM sodium chloride and 10mM calcium chloride) overnight at 4°C. An aliquot of Nickel-Agarose beads (30µl) were washed 2 x 0.5ml wash buffer. The dialysed solution was mixed with the washed Nickel-Agarose beads by inversion at 4°C for 2 hours. The beads were washed 2 x 1ml wash buffer and eluted in 30µl elution buffer (50mM tris HCl pH7.5 with 6M urea and 500mM sodium chloride). The elutions were stored at –80°C until required.

6.3.5 Inhibition of 'Aggrecanase Activity' of Heparin and Zinc Chelator Bound Media Samples from IL-1α Treated Cultures, Against the IGD of Purified Aggrecan (A1D1), by Preincubation with N-TIMP-3 and Recombinant Human TIMP-3

Purified aggrecan (A1D1) was prepared as described in Chapter 2 Section 2.2.6. To aliquots of A1D1 (100µg GAG equivalent) was added 300µl heparin or zinc chelator bound fractions (see Section 5.4.6) with 1/10 volume 10x buffer (20mM tris, 100mM sodium chloride, 10mM calcium chloride, pH 7.5 with 2.5% (v/v) triton) in the absence or presence of human recombinant TIMP-3 (1µg protein) or the recombinant protein comprising the amino-terminal region of human TIMP-3 (N-TIMP-3) (100nM). The mixtures were incubated at 37°C for 24 hours before isolation of the sulphated GAG bearing aggrecan fragments using cetylpyridinium chloride (CPC) precipitation as described in Chapter 5 (Section 5.3.5).

The samples were reconstituted, in Laemmli sample buffer (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed under reducing conditions on 4-12% Tris Glycine gels, electrophoretically transferred and subjected to Western blot analysis with M'Ab BC-3 as described in Chapter 2 Section 2.2.8.

6.4 Results

6.4.1 Western Blot Analysis of TIMP-3 Present in Chondrocytes-Agarose Cultures

In order to determine the effects of culture in serum free conditions and stimulation by IL-1α on the TIMP-3 protein secreted by porcine chondrocytes embedded in agarose, and precultured for 21 days, detergent extracts of the agarose plugs and experimental media samples were analysed by Western blotting using a polyclonal antibody raised against the carboxy-terminal region of TIMP-3.

Samples of recombinant human TIMP-3 (0.5μg protein per lane), detergent extracts of agarose plugs at time zero prior, to treatment in serum free conditions, as well as experimental media samples and detergent extracts of agarose plugs from cultures treated with or without IL-1α for 24 - 120 hours (all at 50μl per lane) were electrophoresed, under reducing and non-reducing conditions, on 12% SDS-PAGE slab gels, electrophoretically transferred and subjected to Western blot analysis using a polyclonal antibody raised against the carboxy-terminal region of TIMP-3. The banding pattern was the same for all time points tested, therefore data is shown for 96 hours treatment time as representative (Figures 6.1 and 6.2).

The antibody to the carboxy-terminal region of the protein detects recombinant human TIMP-3 as a doublet at 24 and 27kD under reducing conditions and a single broad band at ~25kD under non-reducing conditions (Figures 6.2A and 6.1A, respectively). The predicted molecular weight of human TIMP-3 is 21.7kD, however previous analysis by SDS-PAGE has detected bands of the glycosylated protein at 30 and 23kD (Triple Point Biologics data sheet). The recombinant human TIMP-3 bands detected are quite diffuse presumably due to variable glycosylation of the protein.

The predominant bands detected in the detergent extracts of agarose cultures were also a doublet at 24 and 27kD under reducing conditions (Figures 6.2 B and C). Under non-reducing conditions a single broad band at ~25kD is detected (Figures 6.1 B and C). These TIMP-3 bands were detected in equal intensity in detergent extracts of agarose plugs from control and IL-1α treated cultures at all treatment times tested (data for 96 hours shown in Figures 6.1C and 6.2C), as well as at time zero, prior to treatment in serum free conditions (Figures 6.1B and 6.2B).

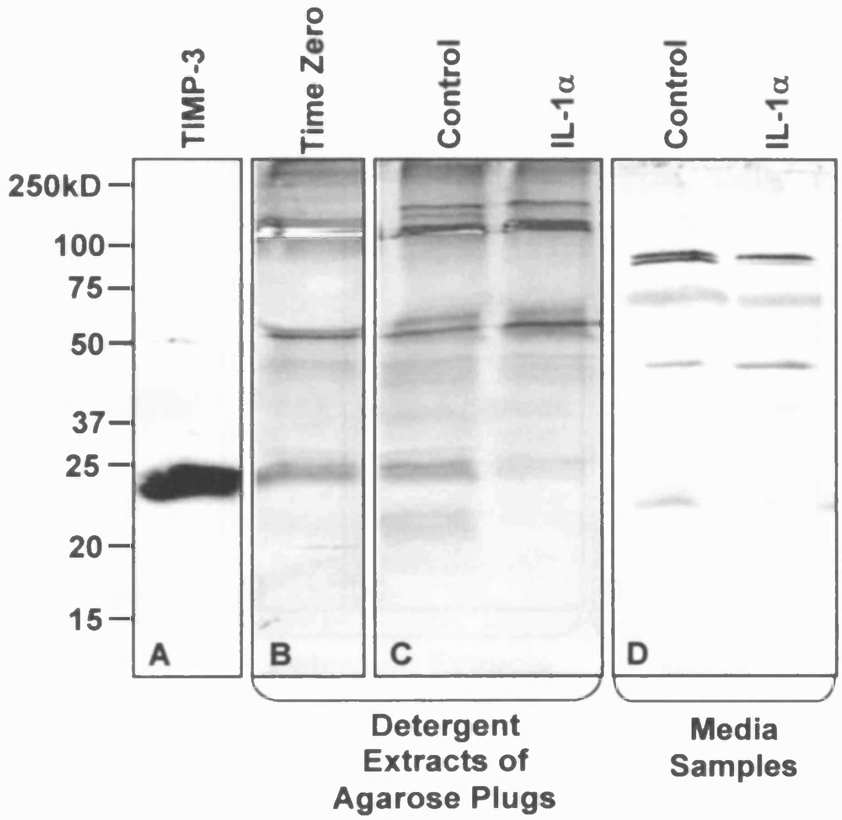


Figure 6.1 Western blot analysis of samples electrophoresed under non-reducing conditions of (A) Recombinant human TIMP-3 (0.5μg protein/lane), (B) Detergent extracts of agarose plugs from cultures at time zero, (C) Detergent extracts of agarose plugs and (D) Media samples from agarose cultures following treatment in the absence (control) or presence of IL-1α (10ng/ml) for 96 hours (all at 50μl/lane).

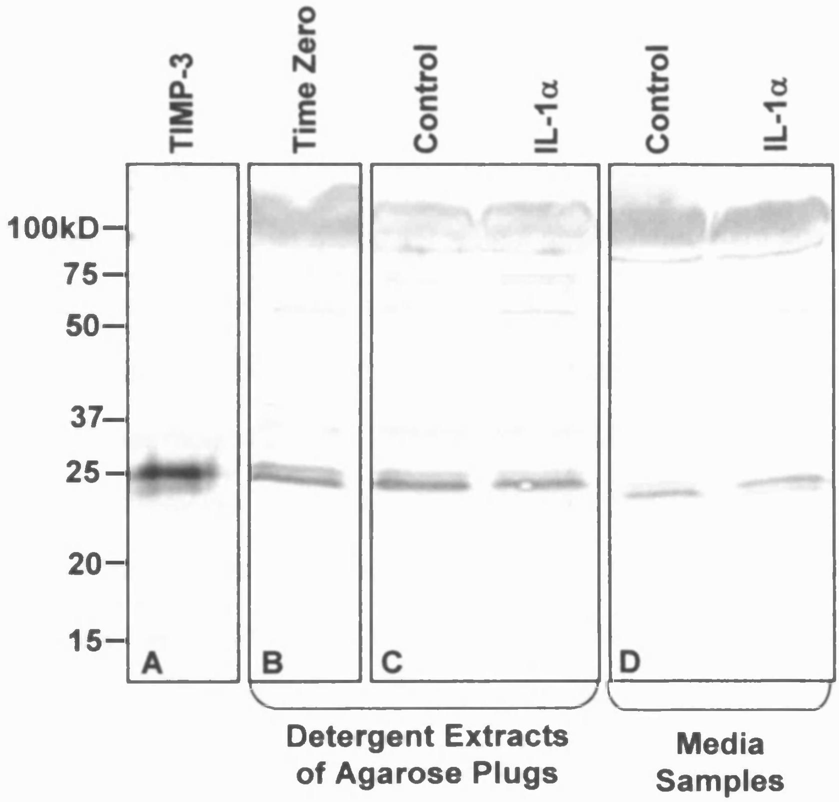


Figure 6.2 Western blot analysis of samples electrophoresed under reducing conditions of (A) Recombinant human TIMP-3 (0.5μg protein/lane), (B) Detergent extracts of agarose plugs from cultures at time zero, (C) Detergent extracts of agarose plugs and (D) Media samples from agarose cultures following treatment in the absence (control) or presence of IL-1α (10ng/ml) for 96 hours (all at 50μl/lane).

The predominant TIMP-3 bands detected in media samples from agarose cultures were a doublet at 24 and 27kD under reducing conditions (Figure 6.2D). Interestingly, the higher molecular weight glycosylated form of TIMP-3 is detected in increased intensity in media samples from IL-1 α treated cultures whereas in the control cultures the lower molecular weight deglycosylated form of TIMP-3 predominates. The same doublet of TIMP-3 bands was detected, at 24 and 27kD, in media samples under non-reducing conditions and here the lower molecular weight non-glycosylated form of the molecule is detected in increased amounts in control cultures compared to IL-1α treated cultures, however, the higher molecular weight glycosylated form does not predominate in the IL-1α treated cultures (Figure 6.1D).

Staining of detergent extracts of agarose plugs and media samples also revealed a complex pattern of higher molecular weight bands indicating TIMP-3 to be associated with various matrix molecules, which may include isoforms of ADAMTS-4 and –5.

In the detergent extracts of agarose plugs under reducing conditions higher molecular weight immunopositive bands were detected in detergent extracts of agarose plugs at 37, 40, 55 and 75kD with a large diffuse band at ~100kD (Figures 6.2 B and C). Under non-reducing conditions bands were detected at 45, 75, 100 and 110kD as well as a broad band at ~55kD (Figures 6.1 B and C). These bands were detected in equal intensity in extracts of agarose plugs from cultures treated in the absence (control) or presence of IL-1α for all time points analysed (data for 96 hours is shown in Figures 6.1C and 6.2C), as well as at time zero prior to treatment in serum free conditions (Figure 6.1B and 6.2B).

The TIMP-3 immunopositive band(s) visualised in the detergent extracts of agarose plugs at ~25kD were detected in apparently increased intensity under reducing conditions compared to non-reducing conditions (Figures 6.2 B and C, and Figures 6.1 B and C, respectively). This indicates that some TIMP-3 interactions may be dissociated under reducing conditions which are still viable under non-reducing conditions.

Interestingly, the 100kD diffuse TIMP-3 immunopositive band detected in detergent extracts of agarose plugs was significantly reduced in intensity under non-reducing conditions (Figures 6.1 B and C) compared to under reducing conditions (Figures 6.2 B and C). This may indicate TIMP-3 associated with a very large molecule unable to pass into the SDS-PAGE gel under non-reducing conditions. The molecular weight of this complex may be massively greater than 100kD as this was the highest molecular weight marker visible of 12% SDS-PAGE slab gels.

In media samples under reducing conditions immunopositive bands were detected at 55 and 90kD as well as a diffuse band at ~100kD (Figure 6.2C). Under non-reducing conditions immunopositive bands were detected at 50 and 75kD with a doublet at 100kD (Figure 6.1C). These bands were detected in equal intensity in experimental media samples from control and IL-1α treated chondrocyte-agarose cultures for all treatment times tested (data shown at 96 hours in Figures 6.2C and 6.1C).

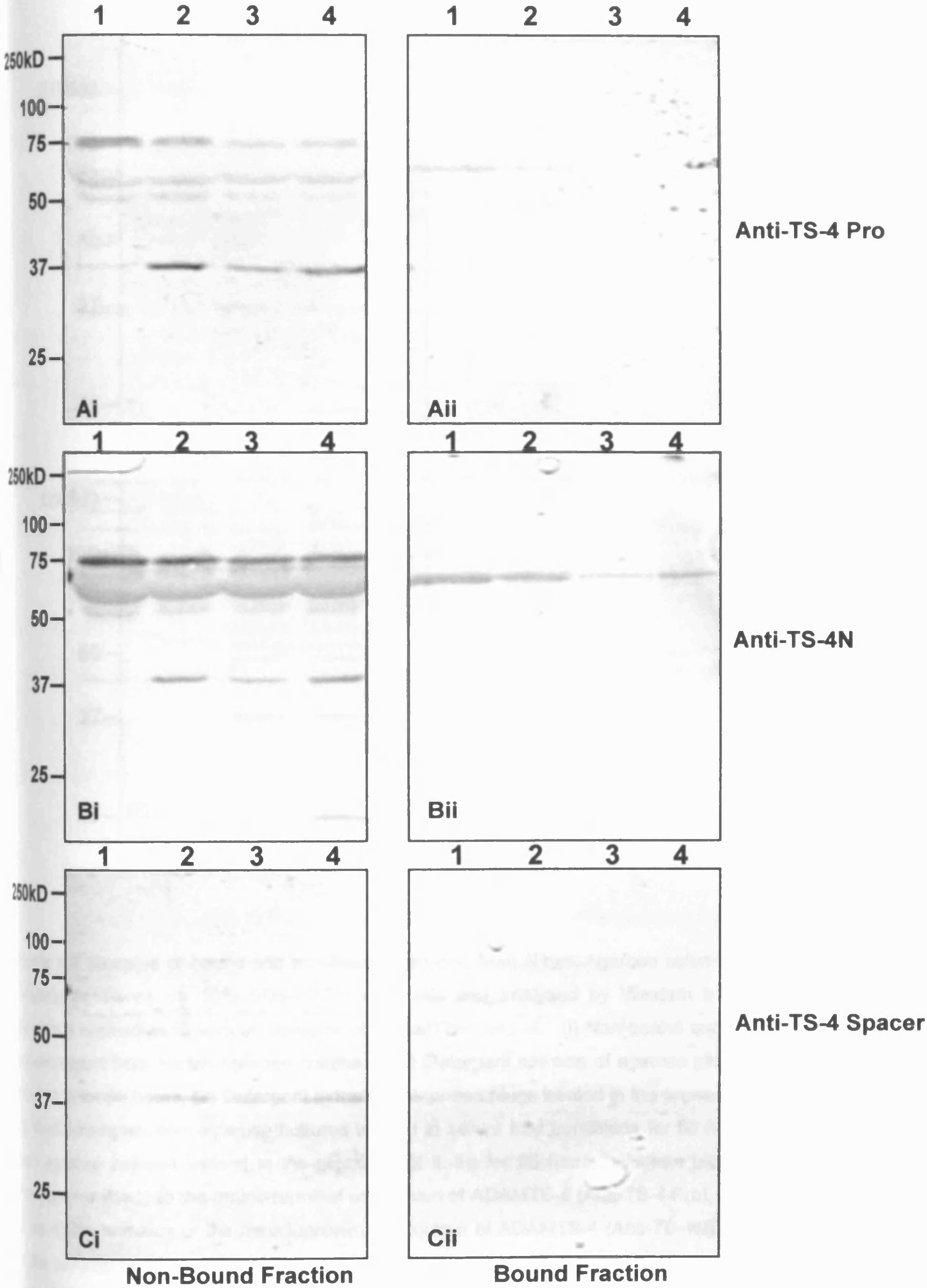
The 50, 75 and 90kD TIMP-3 immunopositive bands detected in media samples under non-reducing conditions were present at markedly decreased intensity under reducing conditions. The 25kD TIMP-3 immunopositive bands detected in media samples were markedly increased under reducing conditions compared to non-reducing conditions. This suggests that under non-reducing conditions TIMP-3 is associated with other molecules resulting in 50, 75 and 90kD immunopositive bands which are dissociated under reducing conditions resulting in detection of apparently increased amounts of free (~25kD) TIMP-3.

As described above, in detergent extracts of agarose plugs, the diffuse 100kD TIMP-3 immunopositive band detected in media samples was markedly decreased in intensity under non-reducing (Figure 6.1D) conditions compared to under reducing conditions (Figure 6.2D). This may indicate TIMP-3 associated with a very large molecule unable to pass into the SDS-PAGE gel under non-reducing conditions. The molecular weight of this complex may be massively greater than 100kD as this was the highest molecular weight marker visible on 12% SDS-PAGE gels.

6.4.2 Isoforms of ADAMTS-4 and –5 which are Bound by a Recombinant Protein Comprising the Amino-Terminal Region of Human TIMP-3 (N-TIMP-3)

In our model chondrocyte-agarose culture system TIMP-3 was detected both sequestered in the matrix and released to the experimental medium. In order to determine whether the TIMP-3 is interacting with isoforms of ADAMTS-4 and -5 identified in the chondrocyte-agarose culture system (see Chapter 5) a recombinant protein comprising the amino terminal region of human TIMP-3 labelled with a His Tag (N-TIMP-3) was used to capture the enzymes. The recombinant TIMP-3 protein was incubated with samples of experimental media or detergent extracts, from cultures treated with and without IL-1α for 96 hours. The N-TIMP-3 and any associated proteins were then retrieved using a Nickel-Agarose column which will bind the His Tag motif of the recombinant protein. The column was eluted and samples of the bound and non-bound fractions were electrophoresed under reducing conditions on 10% SDS-PAGE slab gels and subjected to Western blot analysis using the M'Ab raised against the amino-terminus of the metalloproteinase domain of ADAMTS-4, Anti-TS-4N (characterised in Chapter 5 Sections 5.4.2 and 5.4.3), as well as polyclonal antibodies to the pro- and spacer domains of ADAMTS-4 and –5 (characterised in Chapter 5 Section 5.4.3). The results shown in Figure 6.3 demonstrate interactions between N-TIMP-3 and isoforms of both ADAMTS-4 and -5. The isoforms of ADAMTS-4 and -5 available to bind to the recombinant N-TIMP-3 were those not associated with endogenous TIMP-3 shown to be present in this culture system (Section 6.4.1). N-TIMP-3 may also compete for binding with native TIMP-3.

In the non-bound supernatant from the Nickel-Agarose column the mono- and polyclonal antibodies to the pro-, metalloproteinase and spacer domains of ADAMTS-4 and the polyclonal antibody to the prodomain of ADAMTS-5 detected co-migrating enzyme isoforms at 37kD, which were present in increased intensity where the starting material for the column was experimental media samples and detergent extracts of agarose plugs from plates cultured in the presence of IL-1α compared to where untreated control cultures were used (Figures 6.3 Ai, Bi, Ci and Di).


1 2 3 4
250kD
100
75
50
37
25
Ai
Aii
Anti-TS-4 Pro
Bi
Bii
Anti-TS-4N
Ci
Cii
Anti-TS-4 Spacer
Non-Bound Fraction
Bound Fraction

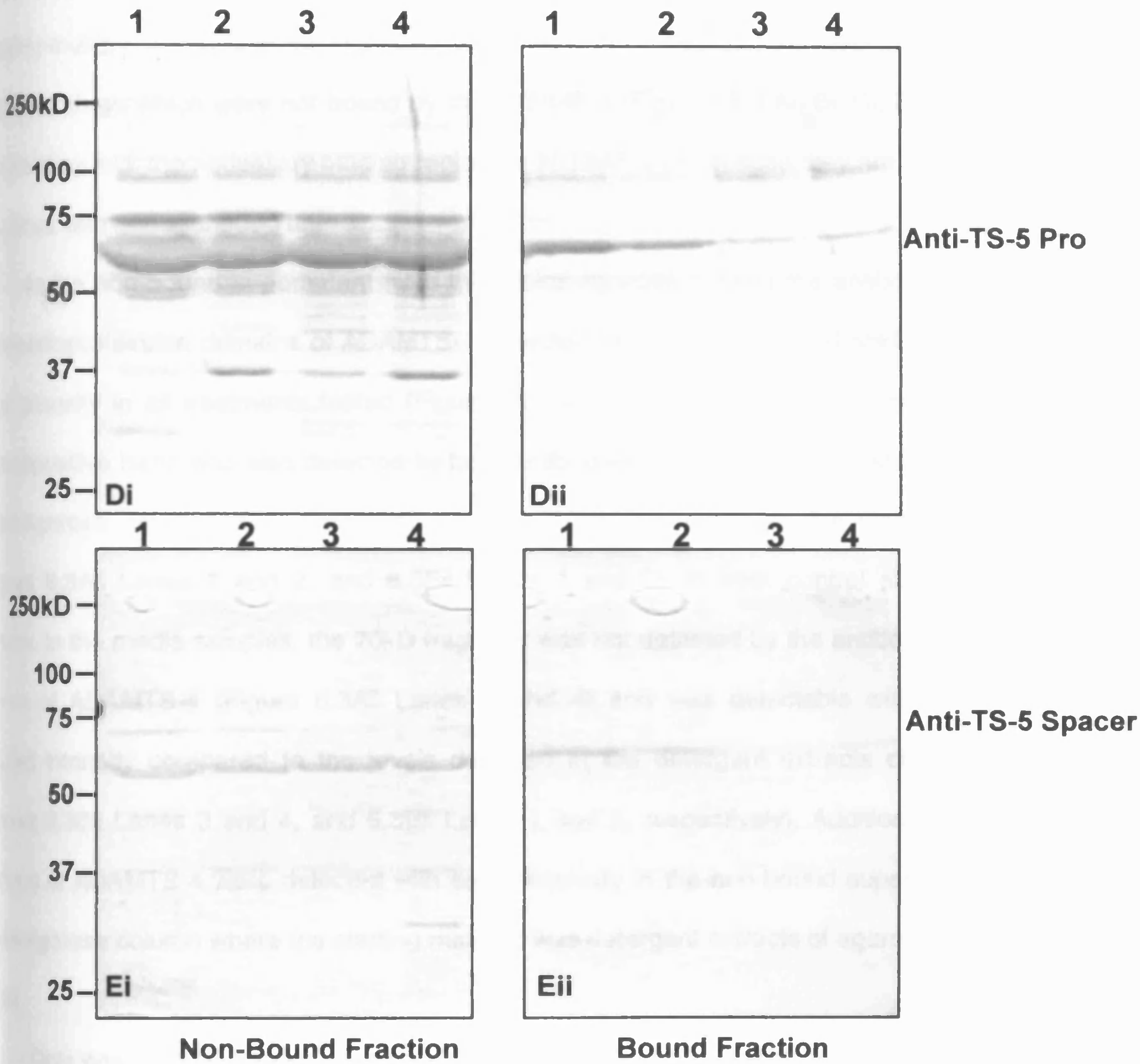


Figure 6.3 Samples of bound and non-bound fractions from Nickel-Agarose column electrophoresed under reducing conditions on 10% SDS-PAGE slab gels and analysed by Western blotting using mono- and polyclonal antibodies to various domains of ADAMTS-4 and -5. (i) Non-bound supernatant, and (ii) Bound column eluent from Nickel-Agarose column of (1) Detergent extracts of agarose plugs treated in serum free conditions for 96 hours, (2) Detergent extracts of agarose plugs treated in the presence of IL-1α for 96 hours, (3) Media samples from agarose cultures treated in serum free conditions for 96 hours, (4) Media samples from agarose cultures treated in the presence of IL-1α for 96 hours. Western blots were probed with (A) Polyclonal antibody to the amino-terminal prodomain of ADAMTS-4 (Anti-TS-4 Pro), (B) Monoclonal antibody to the amino-terminus of the metalloproteinase domain of ADAMTS-4 (Anti-TS-4N), (C) Polyclonal antibody to the carboxy-terminal spacer domain of ADAMTS-4 (Anti-TS-4 Spacer), (D) Polyclonal antibody to the amino-terminal prodomain of ADAMTS-5 (Anti-TS-5 Pro) and (E) Polyclonal antibody to the spacer domain of ADAMTS-5 (Anti-TS-5 Spacer).

Several additional immunopositive bands corresponding to different isoforms of ADAMTS-4 and -5 were also detected in the non-bound supernatant from the Nickel-Agarose column indicating multiple isoforms of the enzymes to be present in media samples and detergent extracts of agarose plugs which were not bound by the N-TIMP-3 (Figures 6.3 Ai, Bi, Ci, Di and Ei) either because they lack the necessary binding region for N-TIMP-3 or because they are already strongly associated with endogenous TIMP-3.

In the non-bound supernatant from the Nickel-Agarose column the antibodies to the pro- and metalloproteinase domains of ADAMTS-4 detected bands at 75, 60 and 55kD, which were of equal intensity in all treatments tested (Figures 6.3Ai Lanes 1-4 and 6.3Bi Lanes 1-4). A single immunopositive band was also detected by both antibodies at 70kD in the bound fraction from the Nickel-Agarose column using detergent extracts of agarose plugs as the starting preparation (Figures 6.3Aii Lanes 1 and 2, and 6.3Bii Lanes 1 and 2), in both control and IL-1α treated cultures. In the media samples, the 70kD fragment was not detected by the antibody to the spacer domain of ADAMTS-4 (Figure 6.3Aii Lanes 3 and 4) and was detectable with Anti-TS-4N at reduced intensity compared to the levels detected in the detergent extracts of agarose plugs (Figures 6.3Bii Lanes 3 and 4, and 6.3Bii Lanes 1 and 2, respectively). Additional 30 and 20kD isoforms of ADAMTS-4 were detected with equal intensity in the non-bound supernatant from the Nickel-Agarose column where the starting material was detergent extracts of agarose plugs (Figure 6.3Bi).

Only weak staining was observed with the antibody to the spacer domain of ADAMTS-4. A 70kD band was detectable in the non-bound fractions which was of equal intensity in all treatments tested (Figure 6.3Ci Lanes 1-4). No ADAMTS-4 isoforms were detectable using the antibody to the spacer domain of the protein in the bound fraction from the Nickel-Agarose column (Figure 6.3Cii Lanes 1-4).

In the non-bound supernatant from the Nickel-Agarose column the polyclonal antibody to the amino-terminal prodomain of ADAMTS-5 detected bands at 100, 75, 60, 55 and 30kD, which were of equal intensity in all treatments tested (Figure 6.3Di Lanes 1-4). In the bound fraction from the Nickel-Agarose column isoforms of ADAMTS-5 were detected with equal intensity at 240 and 100kD using the antibody to the prodomain of the protein (Figure 6.3Dii Lanes 1-4). An additional isoform of ADAMTS-5 was detected at 70kD, which was present in increased intensity in the detergent extracts of agarose plugs compared to equivalent samples of experimental media (Figures 6.3Eii Lanes 1 and 2, and 6.3Eii Lanes 3 and 4, respectively).

The intensity of staining detected with the polyclonal antibody to the spacer domain of ADAMTS-5 was very faint with bands detectable in the non-bound fractions at 70 and 55kD which were of equal intensity in all treatments tested (Figure 6.3Ei Lanes 1-4). A single ADAMTS-5 isoform was detected at 60kD, with equal intensity, in the bound fractions from the Nickel-Agarose column (Figure 6.3Eii).

6.4.3 Inhibition of 'Aggrecanase Activity' of Heparin and Zinc Chelator Bound Media Samples from IL-1α Treated Cultures, Against the IGD of Purified Aggrecan (A1D1), by Preincubation with Recombinant Human N-TIMP-3 and TIMP-3

The heparin and zinc chelator bound media fractions from chondrocyte-agarose cultures treated with IL-1α for 96 hours were assayed for 'aggrecanase activity' against the Glu^{373}-Ala^{374} bond within the interglobular domain of aggrecan (detected using M'Ab BC-3) using purified porcine aggrecan (A1D1) as a substrate, in the presence and absence of a recombinant protein comprising the amino-terminal region of human TIMP-3 (N-TIMP-3) or recombinant human TIMP-3.

The starting preparation of purified A1D1 did not contain any BC-3 positive aggrecan catabolites (Figures 6.4 A and E). However, A1D1 digested with heparin bound or zinc chelator bound media fractions contained BC-3 positive bands ranging in molecular weight from 30 to >250kD (Figures 6.4 B and F, respectively). This indicates that both the heparin and zinc chelator column eluents contain 'aggrecanase activity' directed against the Glu^{373}-Ala^{374} bond within the interglobular domain of aggrecan. This 'IGD aggrecanase activity' appears to be greater in the heparin bound media fractions than in the zinc chelator bound media fractions (Figures 6.4 B and F, respectively). However the activity in the zinc chelator bound fractions varied between digests (data not shown).

Preincubation with N-TIMP-3 or recombinant human TIMP-3 decreased the 'aggrecanase activity' in digests from both the heparin and zinc chelator bound media fractions (Figures 6.4 C and D, and 6.4 G and H, respectively). In conclusion this data indicates that the detectable 'aggrecanase activity' against the Glu^{373}-Ala^{374} bond within the interglobular domain of aggrecan present in these eluents is inhibited by the amino-terminal domain of TIMP-3.

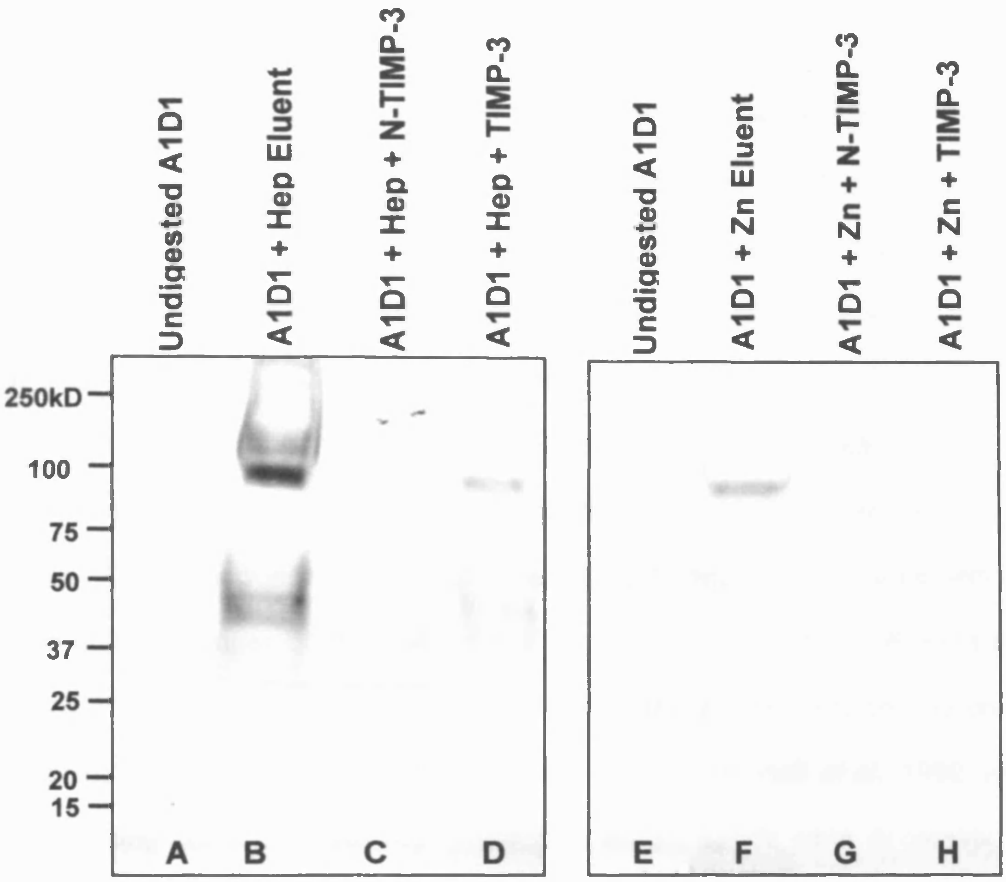


Figure 6.4 Western blot analysis using M'Ab BC-3 of samples of purified A1D1 digested with either heparin or zinc chelator bound media fractions from chondrocyte-agarose cultures treated with IL-1α (10ng/ml) for 96 hours (A) and (E) Purified undigested aggrecan (A1D1), (B) A1D1 digested with Heparin-Sepharose column eluent, (C) A1D1 digested with Heparin-Sepharose column eluent in the presence of N-TIMP-3 (100nM), (D) A1D1 digested with Heparin-Sepharose column eluent in the presence of human recombinant TIMP-3 (1μg), (F) A1D1 digested with Zinc Chelator column eluent, (G) A1D1 digested with Zinc Chelator column eluent in the presence of N-TIMP-3 (100nM), (H) A1D1 digested with Zinc Chelator column eluent in the presence of human recombinant TIMP-3 (1μg). Samples were deglycosylated prior to electrophoretic separation.

6.5 Discussion

The presence of TIMP-3 in the medium and sequestered in the matrix of the model chondrocyte-agarose culture system was assessed using a polyclonal antibody raised against the carboxy-terminal region of the protein. Immunopositive bands were detected at 24 and 27kD (under both reducing and non-reducing conditions) in media samples and detergent extracts of agarose plugs (Figure 6.1B, C and D, and 6.2B, C and D). The bands detected under non-reducing conditions were 'free' TIMP-3 i.e. TIMP-3 not associated with any other molecule. Interestingly, the higher molecular weight glycosylated form of TIMP-3 was detected in increased intensity under reducing conditions in media samples from cultures treated in the presence of IL-1α, whereas in the untreated control cultures the lower molecular weight deglycosylated form of TIMP-3 predominated. The reason for this variation in the glycosylation of TIMP-3 is unknown. In addition to the 'free' TIMP-3, immunopositive bands corresponding to TIMP-3 associated with other matrix molecules were detected under both reducing and non-reducing conditions (Figures 6.1 and 6.2). These molecules may include ADAMTS-4 and -5. The ability of TIMP-3 to bind to components of the extracellular matrix is unique among TIMP family members (Pavloff *et al.*, 1992, and Yu *et al.*, 2000). Under reducing conditions the high molecular weight bands were decreased in intensity compared to under non-reducing conditions. The low molecular weight bands ~25kD were increased in intensity under reducing conditions compared to under non-reducing conditions. This indicates that in this culture system TIMP-3 forms high molecular weight complexes which are only dissociated under reducing conditions.

The above data shows TIMP-3 to be both sequestered in the matrix and released to the medium of chondrocyte-agarose cultures. The amino-terminal region of TIMP-3 has been shown previously to be involved in the inhibition of ADAMTS-4 and -5 (Kashiwagi *et al.*, 2001). Therefore in order to determine whether TIMP-3 is able to interact with the isoforms of ADAMTS-4 and -5 detected in these cultures (described in Chapter 5 Sections 5.4.4, 5.4.5 and 5.4.6) a recombinant protein comprising the amino-terminal region of TIMP-3 labelled with a His Tag was used (N-TIMP-3). The N-TIMP-3 and any associated proteins were bound to Nickel-Agarose then eluted. The bound and non-bound fractions from the Nickel-Agarose column were analysed by Western blotting using the mono- and polyclonal antibodies to domains of ADAMTS-4 and -5 (characterised in Chapter 5 illustrated in Figure 5.1). The results demonstrate an interaction between N-TIMP-3 and isoforms of ADAMTS-4 and -5 (Figure 6.3).

The polyclonal antibodies to the spacer domains of ADAMTS-4 and -5 both detected only very faint immunopositive bands in both the bound and non-bound fractions from the Nickel-Agarose column (Figure 6.3 C and E). This may indicate that N-TIMP-3 stearically hinders binding of the antibodies to the spacer regions of ADAMTS-4 and -5, and may itself interact with the enzymes through their spacer domains. No data is available indicating the region(s) of ADAMTS-4 and -5 which interact with the amino-terminal region of TIMP-3, however it has been suggested to occur through the metalloproteinase domains of the enzymes.

In the non-bound supernatant from the Nickel-Agarose column the antibodies to domains of ADAMTS-4 and the antibody to the prodomain of ADAMTS-5 detected isoforms of the enzymes which co-migrate at 37kD. These 37kD isoforms are detected in increased intensity where the starting material bound to N-TIMP-3 was media samples or detergent extracts of agarose plugs from cultures treated with IL-1α compared to from untreated control cultures. Therefore these 37kD co-migrating isoforms most likely correspond to those detected in the heparin bound media samples described in Chapter 5 Section 5.4.6 and resulting from enzyme catalysis or alternative splicing. These 37kD co-migrating isoforms have been proposed to be the active form of the enzymes since they were detected in increased amounts in heparin bound media samples from IL-1α treated cultures compared to untreated controls (Chapter 5). Interestingly, no 37kD isoforms of ADAMTS-4 or -5 were detected in the detergent extracts of agarose plugs prior to incubation with N-TIMP-3 (Chapter 5 Sections 5.4.4 and 5.4.5). The significance of this observation is at present unknown.

Several additional immunopositive bands corresponding to ADAMTS-4 and -5 were detected in the non-bound supernatant from the Nickel-Agarose column indicating multiple isoforms of the enzymes to be present which are not bound by exogenous N-TIMP-3 (Figures 6.3 Ai, Bi, Ci, Di and Ei). These isoforms of ADAMTS-4 and -5 may lack the domains required for interaction with N-TIMP-3 or may already be associated with endogenous TIMP-3 previously shown to be present in this culture system (Section 6.4.1).

In the bound fractions from the Nickel-Agarose column the major immunopositive band was detected at 70kD by the antibodies to the pro- and metalloproteinase domains of ADAMTS-4 and antibodies to the pro- and spacer domains of ADAMTS-5 (Figures 6.3 Ai, Bi, Di and Ei). The 70kD isoform detected by the antibody to the prodomain of ADAMTS-4 was detected only in the bound fraction from Nickel-Agarose columns where the starting preparation had been detergent extracts of agarose plugs (Figure 6.3Aii). The 70kD isoforms detected by the antibodies to the metalloproteinase domain of ADAMTS-4 and the pro- and spacer domains of ADAMTS-5 were detected in increased intensity in the bound fraction from Nickel-Agarose columns where the starting material was detergent extracts of agarose plugs compared to those where the starting material was media samples (Figures 6.3 Bii, Dii and Eii). The 70kD isoforms of ADAMTS-4 and -5 may correspond to the 70kD zinc chelator bound isoforms of ADAMTS-4 and -5 described in Chapter 5 Section 5.4.6. The 70kD band detected by the antibodies to the metalloproteinase domain of ADAMTS-4 and the spacer domain of ADAMTS-5 may represent the Furin-activated forms of the enzymes (predicted human molecular weights 67.9 and 73.6kD, respectively). The presence of 70kD immunopositive bands detected by the antibodies to the amino-terminal prodomains of ADAMTS-4 and -5 indicates the presence of co-migrating 'active' and 'inactive' isoforms of ADAMTS-4 and -5. Additional immunopositive bands were detected by the antibody to the prodomain of ADAMTS-5 at 100 and 240kD (Figure 6.3Dii), these may correspond to the isoforms of ADAMTS-5 detected at 100 and 240kD in both detergent extracts of agarose plugs and zinc chelator bound fractions of media samples in Chapter 5 Sections 5.4.4, 5.4.5 and 5.4.6. The 100kD isoform may be the zymogen form of ADAMTS-5 (predicted human molecular weight 101.7kD), however, since it was not detected by the antibody to the spacer domain of the enzyme these may be smaller carboxy-terminally truncated isoforms of ADAMTS-5 associated with other matrix molecules such as fibronectin.

This data is in contrast to results already published using N-TIMP-3 and a Nickel-Agarose column as a purification method (Kashiwagi *et al.*, 2004). In the previous study isoforms of ADAMTS-4 were isolated from guanidine HCl extracts of porcine articular cartilage explants cultured with IL-1α for 3 days. The isoforms were detected with an antibody to the metalloproteinase domain of ADAMTS-4 at 46, 40 and 37kD. In the present study none of the co-migrating 37kD isoforms of ADAMTS-4 detected were bound by N-TIMP-3. This may be due to the different methods used to extract ADAMTS-4 from the extracellular matrix. ADAMTS-4 isoforms associated with endogenous TIMP-3 may be dissociated by guanidine HCl extraction, allowing the ADAMTS-4 isoforms to interact with the exogenous N-TIMP-3, whereas the methods employed in the present study use detergent to extract the enzyme isoforms and may not dissociate them from any endogenous TIMP-3.

Media fractions, partially purified via passage over a Heparin-Sepharose column, followed by a Zinc Chelator column, were assayed for 'IGD aggrecanase activity' against the Glu^{373}-Ala^{374} bond within the IGD of aggrecan, using purified porcine aggrecan (A1D1) as a substrate. Digests were preincubated in the presence or absence of the recombinant amino-terminal portion of human TIMP-3 (N-TIMP-3) or human recombinant TIMP-3. A1D1 digested with either heparin or zinc bound media fractions contained BC-3 positive bands ranging in molecular weight from 30->250kD, indicating that the bound fractions from both columns contain 'IGD aggrecanase activity'. The 'IGD aggrecanase activity' was decreased by preincubation with human recombinant TIMP-3 or N-TIMP-3 (Figure 6.4). Therefore the enzyme(s) possessing 'aggrecanase activity' present in the heparin or zinc bound media fractions were inhibited by the amino-terminal region of TIMP-3. ADAMTS-4 and -5 have previously been shown to be inhibited by the amino-terminal region of TIMP-3 (Kashiwagi *et al.*, 2001).

6.6 Summary

- TIMP-3 was present in the culture system of chondrocytes embedded in agarose both in the medium and in detergent extracts of agarose plugs.
- TIMP-3 was present as both 'free' TIMP-3, i.e. not associated with other molecules, and as higher molecular weight complexes some of which are not dissociated even under reducing conditions.
- High molecular weight isoforms of ADAMTS-4 and -5 were shown to interact with the amino-terminal domain of TIMP-3.
- The isoforms of ADAMTS-4 and -5 unable to bind to the amino-terminal region of TIMP-3 may lack the domains necessary for this interaction or may be associated with endogenous TIMP-3.
- Both TIMP-3 and a recombinant protein comprising the amino-terminal domain of TIMP-3 (N-TIMP-3) were able to inhibit the 'IGD aggrecanase activity' of heparin and zinc chelator bound media fractions (described previously in Chapter 5).
- ADAMTS-4 and -5 have been previously shown to be inhibited by both TIMP-3 and N-TIMP-3 (Kashiwagi *et al.*, 2001)

Chapter 7: Investigation of the Effects of Cycloheximide on the Presence of ADAMTS-4 and -5 within Extracellular Matrix Secreted by Chondrocyte-Agarose Cultures

7.1 Introduction

In order to determine whether the isoforms of ADAMTS-4 and –5, and the TIMP-3, present in experimental chondrocyte-agarose cultures result from *de novo* protein synthesis in serum free conditions, experimental cultures were treated with or without of IL-1α in the presence of cycloheximide or its carrier (DMSO). Cycloheximide is an antibiotic produced by Streptomyces Griseus. Its main biological activity is the inhibition of translation via blocking of the peptidyl synthetase activity of eukaryotic ribosomes (Ma *et al.,* 2000, and Lusska *et al.,* 1992) resulting in inhibition of protein synthesis leading, at high concentrations, to subsequent cell growth arrest and cell death. Cycloheximide is widely used at low concentrations for controlled inhibition of protein synthesis and detection of short lived proteins. It has been utilised in previous studies to show that the degradative mechanisms leading to the induction of 'IGD aggrecanase activity' in cartilage explant cultures requires *de novo* protein synthesis (Arner *et al.,* 1998).

In this chapter the effect of cycloheximide treatment on the extracellular matrix synthesised in the model system of chondrocytes embedded in agarose was analysed (using an assay) to determine the proportion of sulphated GAG in the matrix which is released to the medium. The composition of this released sulphated GAG was also analysed using M'Abs, which detect neoepitopes generated by cleavage within the core protein of aggrecan, as described in Chapter 4 (Figure 4.1). Also in Chapter 4 the aggrecanolysis within the IGD detected in the presence of IL-1α was found to be due to the activity of aggrecanases rather than MMPs. Therefore the isoforms of ADAMTS-4 and –5 present in the culture system of chondrocytes embedded in agarose were investigated in Chapter 5. The effects of cycloheximide on the isoforms of ADAMTS-4 and -5 present will be analysed using the mono- and polyclonal antibodies described in Chapter 5 (see Figure 5.1).

Both ADAMTS-4 and –5 have been shown to be inhibited by the amino-terminal portion of TIMP-3 (Kashiwagi *et al.,* 2001) and in this thesis TIMP-3 was shown to be present in this culture system (Chapter 6). Therefore the effects of cycloheximide treatment were analysed using the polyclonal antibody raised against the carboxy-terminal region of TIMP-3 described in Chapter 6.

7.2 Materials

- *Dimethyl sulphonyl oxide (DMSO) was obtained from Sigma Aldrich, Poole, Dorset, UK.*
- *Cycloheximide in DMSO was obtained from Sigma Aldrich, Poole, Dorset, UK.*
- *Monoclonal antibody Anti-TS-4N was produced by Dr. Clare Hughes and Dr. Chris Little using methods described previously (Hughes et al., 1995).*
- *Polyclonal antibodies Anti-ADAMTS-4 Prodomain (RP2-ADAMTS-4), Anti-ADAMTS-4 Spacer domain (RP1-ADAMTS-4), Anti-ADAMTS-5 Prodomain (RP2-ADAMTS-5) and Anti-ADAMTS-5 Spacer domain (RP1-ADAMTS-5) were all obtained from Triple Point Biologics Inc., Forest Grove, OR, US.*
- *Horseradish peroxidase-linked goat anti-mouse antibody was obtained from Amersham, Buckinghamshire, UK.*
- *Horseradish peroxidase-linked goat anti-rabbit secondary antibody was obtained from Amersham, Buckinghamshire, UK.*
- *Blocking agent was obtained from Amersham, Buckinghamshire, UK.*
- *Enhanced Chemiluminescence (ECL) Western blotting Detection reagent was obtained from Amersham, Buckinghamshire, UK.*
- *Hyperfilm ECL was obtained from Amersham, Buckinghamshire, UK.*
- *Recombinant human ADAMTS-4 and –5 were a kind gift from Dr. Carl Flannery, Wyeth, Boston, US.*
- *All other reagents are listed in Chapter 2 Section 2.1, Chapter 4 Section 4.2 and Chapter 6 Section 6.2.*

7.3 Methods

7.3.1 Treatment of Chondrocyte-Agarose Cultures with Cycloheximide in the Presence or Absence of IL-1α

Porcine articular chondrocytes were isolated, embedded in agarose and precultured as described in Chapter 2 Sections 2.2.1 and 2.2.2. Following the 21 day preculture period the plates were washed 3 x 20 minutes in serum free DMEM with 50μg/ml gentamicin before culture in serum free DMEM with 50μg/ml gentamicin and 25μg/ml Phosphitan™C with either 5μg/ml cycloheximide in Dimethyl Sulphonyl Oxide (DMSO) or DMSO alone in the absence (control) or presence of 10ng/ml human recombinant Interleukin-1α (IL-1α). Each treatment was performed on triplicate plates. Following the culture period the medium was removed and stored along with the agarose plugs at -80°C for later analysis.

7.3.2 Analysis of Experimental Medium Collected Following 96 hours Treatment with or without Cycloheximide in the Presence or Absence of IL-1α

The metabolic activity of the chondrocytes present in the chondrocyte-agarose cultures was analysed using the Lactate assay as described in Chapter 2 Section 2.2.5. Proteoglycans present in the agarose plugs were extracted as described in Chapter 2 Section 2.2.3. The concentration of sulphated GAG released to the culture medium, and present in the guanidine extracts and alkaline β-eliminations was analysed using the DMMB assay described in Chapter 2 Section 2.2.4. Western blot analysis was carried out on media samples and guanidine extracts as described in Chapter 2 Section 2.2.8. Positive controls for these analyses were prepared as described in Chapter 2 Sections 2.2.6 and 2.2.7.

7.3.3 Western Blot Analysis of ADAMTS-4 and -5 Isoforms Present in Media Samples and Detergent Extracts of Agarose Plugs Detected Using Enhanced Chemiluminescence (ECL)

Heparin and zinc chelator bound media fractions and detergent extracts of agarose plugs (prepared in Chapter 4 Section 4.3.1 and Chapter 2 Sections 2.2.9 and 2.2.10) (all 50µl per lane) along with recombinant human ADAMTS-4 and -5 (0.5µg per lane) were prepared in Laemmli sample buffer (Laemmli 1970) containing 10% (v/v) β-mercaptoethanol and electrophoresed in duplicate on 10% SDS-PAGE slab gels in running buffer. The gels were then transferred onto Nitrocellulose membrane (0.22µm) in transfer buffer at 100V for 60 minutes. Following their electrophoretic transfer the membranes were subjected to Western blot analysis as described in Chapter 5 (Section 5.3.2).

7.3.4 Western Blot Analysis of TIMP-3

Media samples and detergent extracts of agarose plugs (all 50µl per lane) along with recombinant human TIMP-3 (1µg protein per lane) were electrophoresed, transferred and subjected to Western blot analysis as described in Chapter 6 Section 6.3.3.

7.4 Results

7.4.1 Analysis of Lactate and Sulphated GAG Released from Cultures During Treatment Time

Media samples were analysed using the Lactate assay kit. The results of this analysis showed that treatment with cycloheximide and its carrier (DMSO) to have no effect on the metabolic activity of the chondrocytes present in the chondrocyte-agarose culture system (results not shown). Therefore at the concentrations utilised in these experiments cycloheximide was not causing apoptosis and any effects noted were the result of inhibition of *de novo* protein synthesis.

In order to determine the effect of cycloheximide on the proportion of sulphated GAG released to the medium during the 96 hours experimental treatment time the DMMB assay was utilised. Sulphated GAG was measured in the experimental medium, guanidine extracts of agarose plugs and alkaline β-eliminated extracts of agarose plugs. The concentration of sulphated GAG in the media, guanidine extracts and alkaline β-eliminations was adjusted to per plate and the raw results are shown in Table 7.1.

Statistical analysis of the data obtained for the percentage of the total sulphated GAG released to the medium during the 96 hours treatment time was carried out using Minitab 1.3. Since specific comparisons were required a series of paired t-tests were carried out. Cultures were compared which had been treated with carrier (DMSO) in the absence (control) or presence of IL-1α and treated with cycloheximide in the absence (control) or presence of IL-1α. The results of these analyses are shown in Table 7.2.

Table 7.1 Tabulated results of three separate experiments on triplicate plates, giving a total n of 9, showing the mean GAG (µg/plate) released from cultures treated with cycloheximide (CHX [5µg/ml]) or carrier (DMSO) in the absence (control) or presence of IL-1α (10ng/ml). The GAG released was measured in the culture medium, in guanidine extracts of the agarose plugs and in alkaline β-eliminations of agarose plugs following guanidine extraction. From the results of concentration of sulphated GAG in each of these samples mean results for the total GAG (µg) per plate was calculated and thus the percentage of the total GAG released to the culture medium during the 96 hour treatment time (%).

TREATMENT	GAG (µg/PLATE)				PERCENTAGE OF TOTAL GAG RELEASED TO MEDIUM (%)
	MEDIUM	GUANIDINE EXTRACT	ALKALINE β-ELIMINATION	TOTAL	
CONTROL + DMSO	88.82	104.6	204.88	398.3	22.3
IL-1α + DMSO	264.20	0.0	38.09	302.3	87.4
CONTROL + CHX	64.14	95.3	211.31	370.8	17.2
IL-1α + CHX	92.18	109.1	181.21	382.5	24.1
CONTROL + DMSO	305.44	816.76	299.2	1421.4	21.49
IL-1α + DMSO	553.53	166.14	120.4	840.1	65.89
CONTROL + CHX	172.74	448.71	165.6	787.1	21.95
IL-1α + CHX	181.09	388.07	86.3	655.5	27.63
CONTROL + DMSO	150.92	216.48	201.3	568.7	26.54
IL-1α + DMSO	330.77	110.03	40.1	480.9	68.78
CONTROL + CHX	57.01	128.99	217.9	403.9	14.15
IL-1α + CHX	67.78	142.82	195.6	406.2	16.69

Table 7.2 Tabulated results of paired t-tests with 95% confidence intervals of the mean for the raw data tabulated in Table 7.1. The data shows the mean percentage of the total GAG released to the medium and the p-value obtained when comparing the pairs of data, from cultures treated with cycloheximide (CHX [5µg/ml]) or its carrier (DMSO) in the absence (control) or presence of IL-1α (10ng/ml) for 96 hours.

TREATMENT	MEAN PERCENTAGE OF TOTAL GAG RELEASED TO MEDIUM FROM 3 EXPERIMENTS IN TABLE 7.1 (%)	P-VALUE
CONTROL + DMSO	23.44	0.02
VS		
IL-1α + DMSO	74.02	
CONTROL + CHX	17.8	0.059
VS		
IL-1α + CHX	22.81	

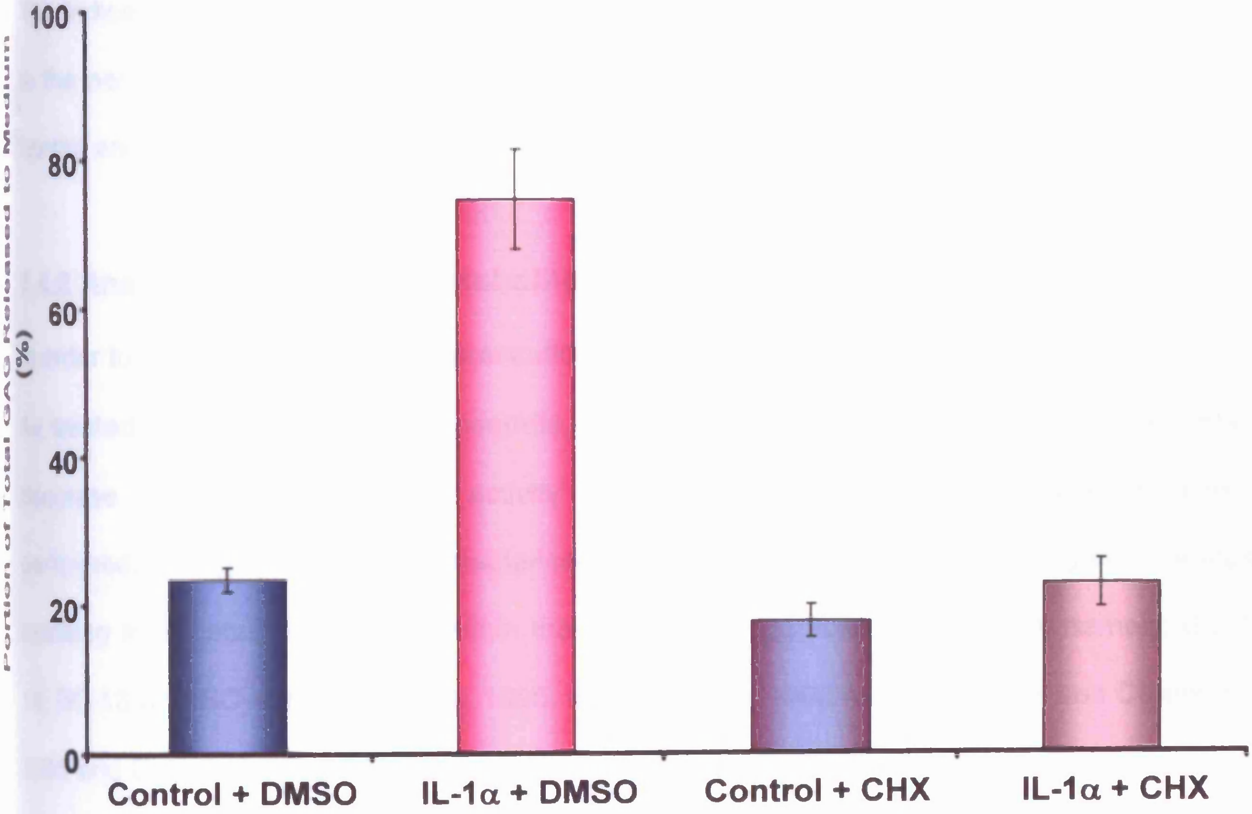


Figure 7.1 Histogram of the mean values for the percentage of the total sulphated GAG released to the culture medium in cultures treated with cycloheximide (CHX [5µg/ml]) or its carrier (DMSO) in the absence (control) or presence of IL-1α (10ng/ml) for 96 hours. Errors shown are Standard Errors.

In control cultures in the presence of carrier (DMSO) ~23% of the total GAG was released to the culture medium during the 96 hours treatment time (Tables 7.1 and 7.2 and Figure 7.1). The release of sulphated GAG to the medium was increased in cultures treated with IL-1α to ~74% of the total GAG present (Table 7.1 and 7.2 and Figure 7.1). A p-value of 0.02 was obtained from a paired t-test comparing cultures treated with or without IL-1α in the presence of DMSO indicating a statistically significant increase in the sulphated GAG released to the culture medium of IL-1α treated cultures compared to the percentage release seen in control cultures.

In contrast, in control and IL-1α treated cultures in the presence of cycloheximide (CHX) ~18 and 23%, respectively, of the total GAG was released to the medium during the 96 hours treatment time (Table 7.1 and 7.2 and Figure 7.1). A p-value of 0.05 was obtained from a paired t-test comparing cultures treated with and without IL-1 in the presence of cycloheximide (Table 7.2). This indicated that in the presence of cycloheximide there was no statistically significant difference in the percentage of the total GAG released to the medium during the treatment time between control and IL-1α treated cultures.

7.4.2 Analysis of Aggrecan Catabolites by Western Blotting

In order to determine whether the prevention of the release of sulphated GAG to the medium of IL-1α treated cultures, compared to controls, caused by treatment with cycloheximide equated to a decrease in detectable enzyme activity against aggrecan a series of Western blots were performed, using previously characterised M'Abs, which specifically recognise neoepitopes resulting from catalytic cleavage within the IGD of the aggrecan core protein, namely; BC-3, BC-14, BC-13 and BC-4 (Hughes *et al.,* 1995, Hughes *et al.,* 1992 and for reviews see Caterson *et al.,* 1995 and Caterson *et al.,* 2000).

Deglycosylated samples of media and guanidine extracts of agarose plugs from control cultures treated with or without cycloheximide and IL-1α treated cultures with or without cycloheximide were electrophoresed, alongside purified aggrecan (A1D1) digested with recombinant human ADAMTS-4 or MMP-13, under reducing conditions on 4–12% SDS-PAGE gels and subjected to Western blot analysis. The results of these analyses are shown in Figures 7.2 and 7.3.

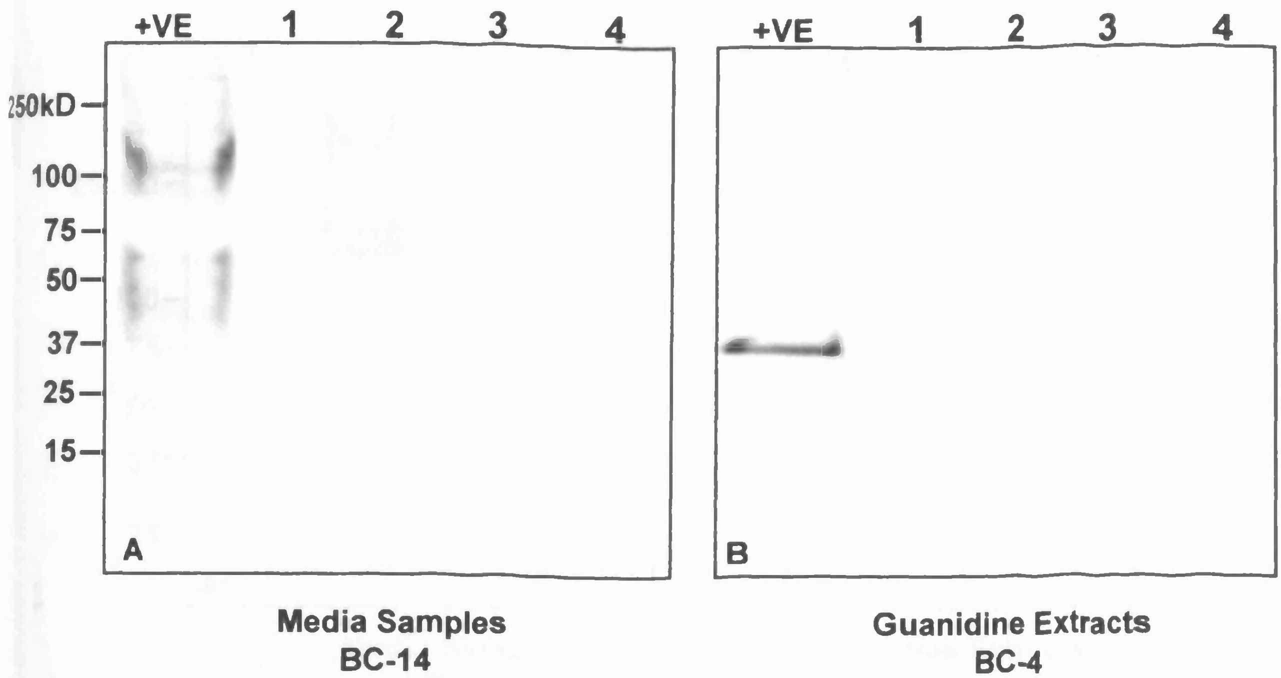


Figure 7.2 Western blot analyses of MMP-generated aggrecan metabolites containing the IGD neoepitopes (A) ^{342}FFGV... detected in deglycosylated media samples by M'Ab BC-14 (20μg GAG equivalent per lane) and (B)...DIPEN^{341} detected in deglycosylated guanidine extracts by M'Ab BC-4 (20μg GAG equivalent per lane). Western blot analysis of deglycosylated media samples and guanidine extracts from cultures treated for 96 hours (1) Control cultures + DMSO, (2) IL-1α (10ng/ml) + DMSO, (3) Control + cycloheximide (CHX [5μg/ml]) and (4) IL-1α (10ng/ml) + cycloheximide (CHX [5μgml]). Positive controls (+VE) were deglycosylated samples of purified porcine aggrecan (A1D1) digested with recombinant human MMP-13 (20μg GAG equivalent per lane).

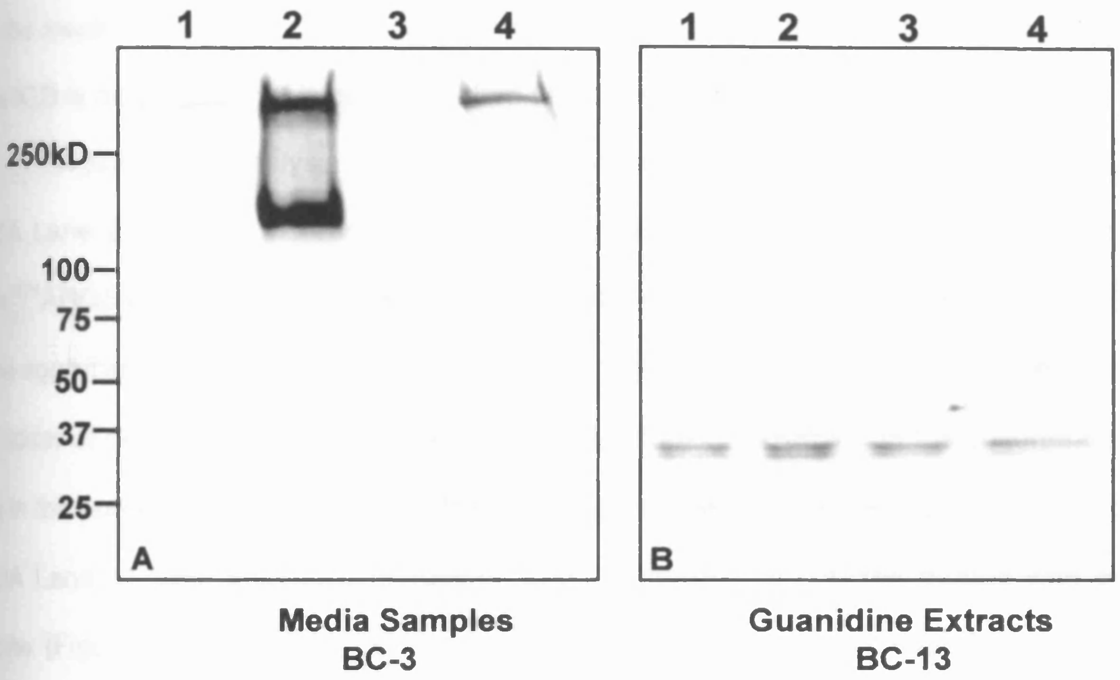


Figure 7.3 Western blot analyses of aggrecanase-generated aggrecan metabolites containing the IGD neoepitopes (A) ^{374}ARGSV... detected in deglycosylated media samples by M'Ab BC-3 (20µg GAG equivalent per lane) and (B)...NITEGE^{373} detected in deglycosylated guanidine extracts by M'Ab BC-13 (20µg GAG equivalent per lane). Western blot analysis of deglycosylated media samples and guanidine extracts from cultures treated for 96 hours (1) Control + DMSO, (2) IL-1α (10ng/ml) + DMSO, (3) Control + cycloheximide (CHX [5µg/ml]) and (4) IL-1α (10ng/ml) + cycloheximide (CHX [5µg/ml]). Positive control of purified aggrecan (A1D1) digested with recombinant human ADAMTS-4 not shown.

Western blot analysis was carried out using M'Abs BC-14 and BC-4 to detect MMP-generated aggrecan catabolites (Figures 7.2 A and B, respectively) and BC-3 and BC-13 to detect aggrecanase-generated aggrecan catabolites (Figures 7.3 A and B, respectively). The Western blots shown in Figures 7.2 A and B demonstrated no MMP-generated aggrecan catabolites in any of the treatments tested. Thus it may be concluded that no MMP activity against aggrecan within the IGD is detectable in this culture system under the conditions used.

Western blot analysis of deglycosylated media samples from IL-1α treated cultures (Figure 7.3A Lane 2) showed increased levels of aggrecanase-generated aggrecan metabolites bearing the ^{374}ARGSV... neoepitope compared to cultures treated with carrier alone (Figure 7.3A Lane 1). The aggrecan catabolites detected in the IL-1α treated cultures were of high molecular weight, with a ladder of bands being detected between >250 – 150kD. However, in the cultures treated with IL-1α in the presence of cycloheximide levels of aggrecanase-generated aggrecan catabolites (Figure 7.3A Lane 4) were increased marginally when compared to cultures treated with cycloheximide alone (Figure 7.3A Lane 3), but this staining was significantly decreased compared to cultures treated with IL-1α and carrier (Figure 7.3A Lane 2). This data indicates that in the presence of cycloheximide IL-1α induced 'IGD aggrecanase activity' is markedly reduced, this correlating with the analysis of sulphated GAG released to the medium shown in Figure 7.1. Western blot analysis showed aggrecan metabolites bearing the ...NITEGE^{373} neoepitope detected by M'Ab BC-13 to be present in equal amounts in guanidine extracts of agarose plugs from all cultures tested (Figure 7.3B).

7.4.3 Western Blot Analyses of ADAMTS–4 and –5 Isoforms Present in Detergent Extracts of Agarose Plugs Following the Experimental Period

In order to determine whether the presence of cycloheximide during the treatment period in serum free medium affected the generation of isoforms of ADAMTS-4 and –5, detergent extraction of the agarose plugs was carried out. Aliquots of the detergent extracts (50µl per lane) were electrophoresed under reducing conditions on 10% SDS-PAGE slab gels and subjected to Western blot analysis with newly characterised monoclonal antibody Anti-TS-4N (characterised in Chapter 5 Section 5.4.1, 5.4.2 and 5.4.3) as well as polyclonal antibodies to the pro- and spacer domains of ADAMTS-4 and –5 (characterised in Chapter 5 Sections 5.4.3). Results from this analysis (Figures 7.4 A-E) showed a similar set of banding patterns whether or not control or IL-1α treated cultures were incubated in the presence or absence of cycloheximide. However, the intensity of staining for some of these bands was reduced in the presence of cycloheximide.

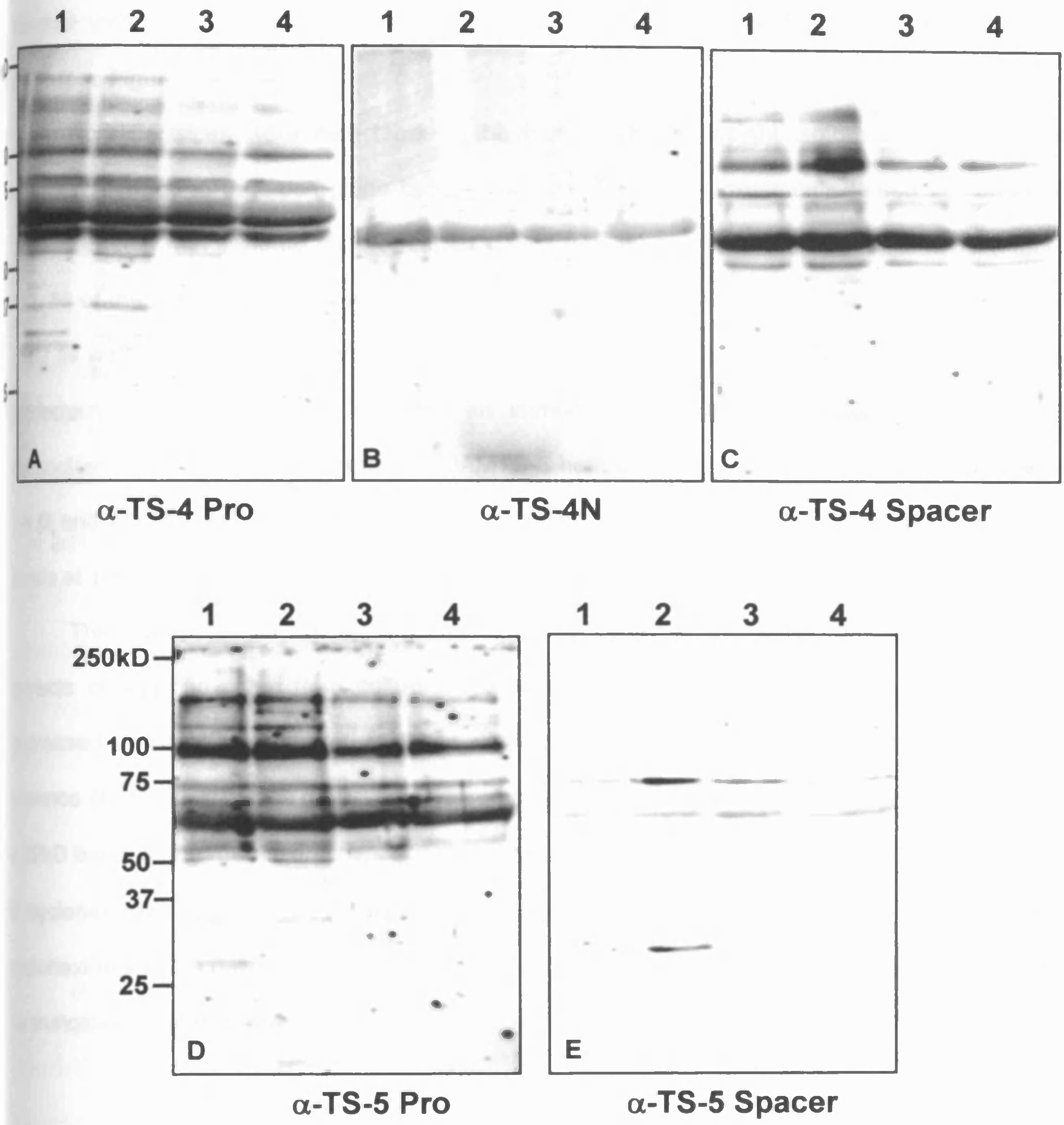


Figure 7.4 Western blot analyses of detergent extracts of agarose plugs (50μl/lane) from cultures treated for 96 hours with (1) Control + DMSO, (2) IL-1α (10ng/ml) + DMSO, (3) Control + cycloheximide (CHX [5μg/ml]) and (4) IL-1α (10ng/ml) + cycloheximide (CHX [5μg/ml]). Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (α-TS-4 Pro), (B) The amino-terminal end of the metalloproteinase domain of ADAMTS-4 (α-TS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (α-TS-4 Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (α-TS-5 Pro) and (E) The spacer domain of ADAMTS-5 (α-TS-5 Spacer).

he series of bands detected are the same as those seen previously for cultures treated in the bsence (control) or presence of IL-1α for 96 hours described in Chapter 5 (Sections 5.4.4 and 4.5) (see Figure 7.4).

No differences were detectable in the high molecular weight isoforms present in the etergent extracts of agarose plugs between any of the culture treatments tested i.e. between ltures treated with cycloheximide or carrier (DMSO) in the presence or absence of IL-1α (Figure 4). All of the antibodies raised against sequences in ADAMTS-4 detected bands at 100, 75 and 5kD as well as a broad band at ~60kD (Figures 7.4 A, B and C). The polyclonal antibody to the rodomain of ADAMTS-4 also detected an immunopositive band at 240kD. The polyclonal ntibodies raised against sequences in ADAMTS-5 both detected bands at 75 and 60kD (Figures 4 D and E). The antibody to the prodomain of ADAMTS-5 also detected high molecular weight ands at 100 and 250kD (Figure 7.4D).

The low molecular weight isoforms detected by all of the antibodies in the detergent xtracts of agarose plugs from cultures treated in the presence of cycloheximide are greatly ecreased in intensity compared to those detected in the extracts from cultures treated in the bsence of cycloheximide. The antibody raised against the spacer domain of ADAMTS-5 detected 32kD band in detergent extracts of agarose plugs from cultures treated with IL-1α in the absence f cycloheximide (Figure 7.4E). This band is absent from control cultures and those treated with cloheximide. This data indicates *de novo* protein synthesis in serum free medium to be required r truncation ADAMTS-4 and -5 resulting in small isoforms.

.4.4 Western Blot Analysis of ADAMTS-4 and -5 Isoforms Present in the perimental Medium of Control and IL-1α Treated Cultures in the Presence and bsence of Cycloheximide

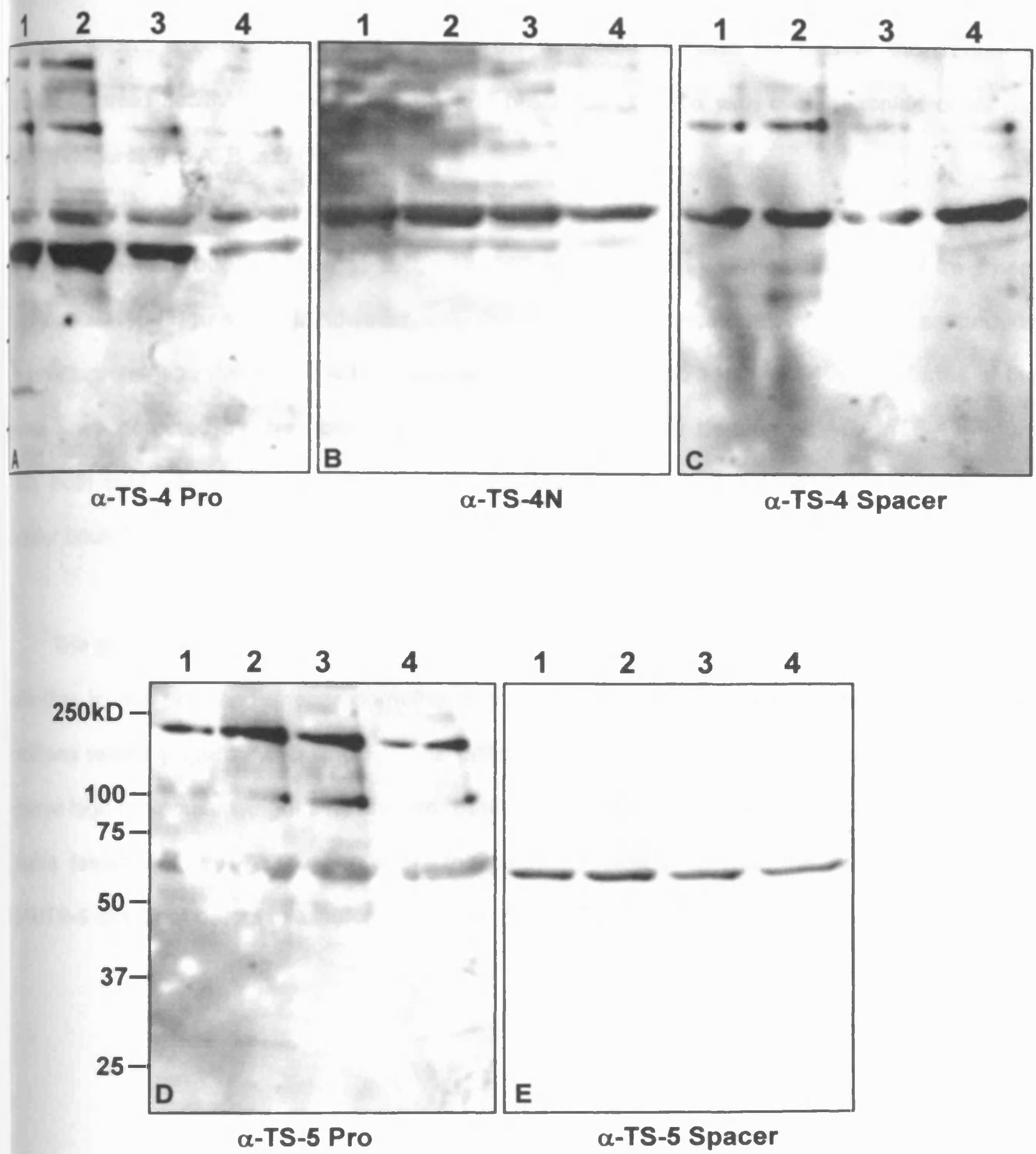
n order to determine whether cycloheximide affected the production of isoforms of ADAMTS-4 and 5 released to the medium of cultures treated in the absence (control) or presence of IL-1α scribed in Chapter 5 (Section 5.4.6) a series of similar Western blots were carried out on media amples partially purified via passage over a Heparin-Sepharose column. The Heparin-Sepharose lumn supernatant was further purified via passage over a Zinc Chelator column, and bound actions from both columns were taken for analysis (as described in Chapter 5 Section 5.4.6).

Aliquots of the eluents (50μl per lane) were electrophoresed under reducing conditions on 0% SDS-PAGE slab gels and subjected to Western blot analysis with newly characterised M'Ab nti-TS-4N (characterised in Chapter 5 Sections 5.4.2 and 5.4.3) as well as polyclonal antibodies o the pro- and spacer domains of ADAMTS-4 and –5 (characterised in Chapter 5 Section 5.4.3). he resulting blots showed multiple isoforms of ADAMTS-4 and –5 to be present in the medium om cultures treated with cycloheximide or carrier (DMSO) in the absence (control) and presence f IL-1α (Figures 7.5 and 7.6).

- **Zinc Chelator Bound Media Fractions**

Media Samples Partially Purified via Passage over Heparin-Sepharose and Bound by a Zinc Chelator Column

he zinc chelator bound isoforms of ADAMTS-4 and -5 detected showed no differences between e various treatments tested (Figure 7.5) i.e. cultures treated in the absence (control) or presence f IL-1α with cycloheximide or carrier (DMSO). As expected in the control and IL-1α treated ultures the isoforms of ADAMTS-4 and -5 detected are the same as those described in Chapter 5 ection 5.4.6. Staining revealed a complex pattern of bands with the polyclonal antibodies to the rodomains of ADAMTS-4 and -5 (Figures 7.5 A and D), with more simplistic patterns appearing ith the M'Ab Anti-TS-4N and the polyclonal antibodies to the spacer domains of ADAMTS-4 and - (Figures 7.5 C and E).



igure 7.5 Western blot analyses of ADAMTS-4 and –5 isoforms bound to Zinc Chelator column and eluted 135mM imidazole (50μl per lane) from media samples treated for 96 hours with (1) Control + DMSO, (2) IL-α (10ng/ml) + DMSO, (3) Control + cycloheximide (CHX [5μg/ml]) and (4) IL-1α (10ng/ml) + cycloheximide HX [5μg/ml]). Western blots were probed with antibodies to (A) The amino-terminal prodomain of DAMTS-4 (α-TS-4 Pro), (B) The amino-terminal end of the metalloproteinase domain of ADAMTS-4 (α-TS-N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (α-TS-4 Spacer), (D) The amino-terminal odomain of ADAMTS-5 (α-TS-5 Pro) and (E) The spacer domain of ADAMTS-5 (α-TS-5 Spacer).

The predominant ~70kD zinc chelator bound isoform of ADAMTS-4 was detected by all of the antibodies raised against domains of the enzyme in media samples from all of the treatment conditions tested i.e. the absence (control) and presence of IL-1α with cycloheximide or carrier (DMSO) (Figures 7.5 A, B and C).

The polyclonal antibody to the prodomain of ADAMTS-4 detects numerous high molecular weight zinc chelator bound bands at 100, 110, 240 and 260kD in the medium from all treatment conditions tested (Figure 7.5A). However, only the 100kD isoform was detected by the antibody to the metalloproteinase domain of ADAMTS-4 (Figure 7.5B) and the 110 and 240kD isoforms of the protein were detected by the polyclonal antibody to the spacer domain of ADAMTS-4 (Figure 7.5C). Both antibodies to the pro- and metalloproteinase domains of ADAMTS-4 detected a zinc chelator bound band of 55kD in medium from all experimental conditions tested (Figure 7.5 A and B).

The predominant ~70kD zinc chelator bound isoform of ADAMTS-5 is detected by both the antibodies to the pro- and spacer domains of the enzyme in media samples from all treatment conditions tested (Figure 7.5 D and E). The polyclonal antibody to the pro-domain of ADAMTS-5 detected high molecular weight zinc chelator bound bands, at 100 and 240kD, in the media from all cultures tested (Figure 7.5D). However, the antibody raised against the spacer domain of ADAMTS-5 does not detect this isoform of the enzyme (Figure 7.5E).

- **Heparin Bound Media Fractions**

Media Samples Partially Purified via Passage over a Heparin-Sepharose Column

The immunopositive bands detected in control and IL-1α treated cultures using antibodies to various domains of ADAMTS-4 and -5 were the same as those previously detected under experimental conditions which did not include DMSO (Chapter 5 Section 5.4.6). Hence any changes detected in the presence of cycloheximide were due to the effects of the cycloheximide itself and not its carrier (DMSO). In these systems increased prevalence of co-migrating 37kD isoforms were detected by antibodies to different domains of ADAMTS-4 and -5 in IL-1α treated cultures compared to controls (Chapter 5 Section 5.4.6 Figures 5.8 A-E and Figures 7.6 A-E Lanes 1 and 2) with an additional 55kD isoform of ADAMTS-4 being detected by the antibody to the spacer domain of the protein (Chapter 5 Section 5.4.6 Figure 5.8C and Figure 7.6C Lanes 1 and 2). Interestingly, in the cultures treated with cycloheximide there was no increase in the 37kD isoforms of ADAMTS-4 and -5 in the IL-1α treated cultures (Figures 7.6 A-E Lane 4) compared to control cultures treated with cycloheximide (Figures 7.6 A-E Lane 2).

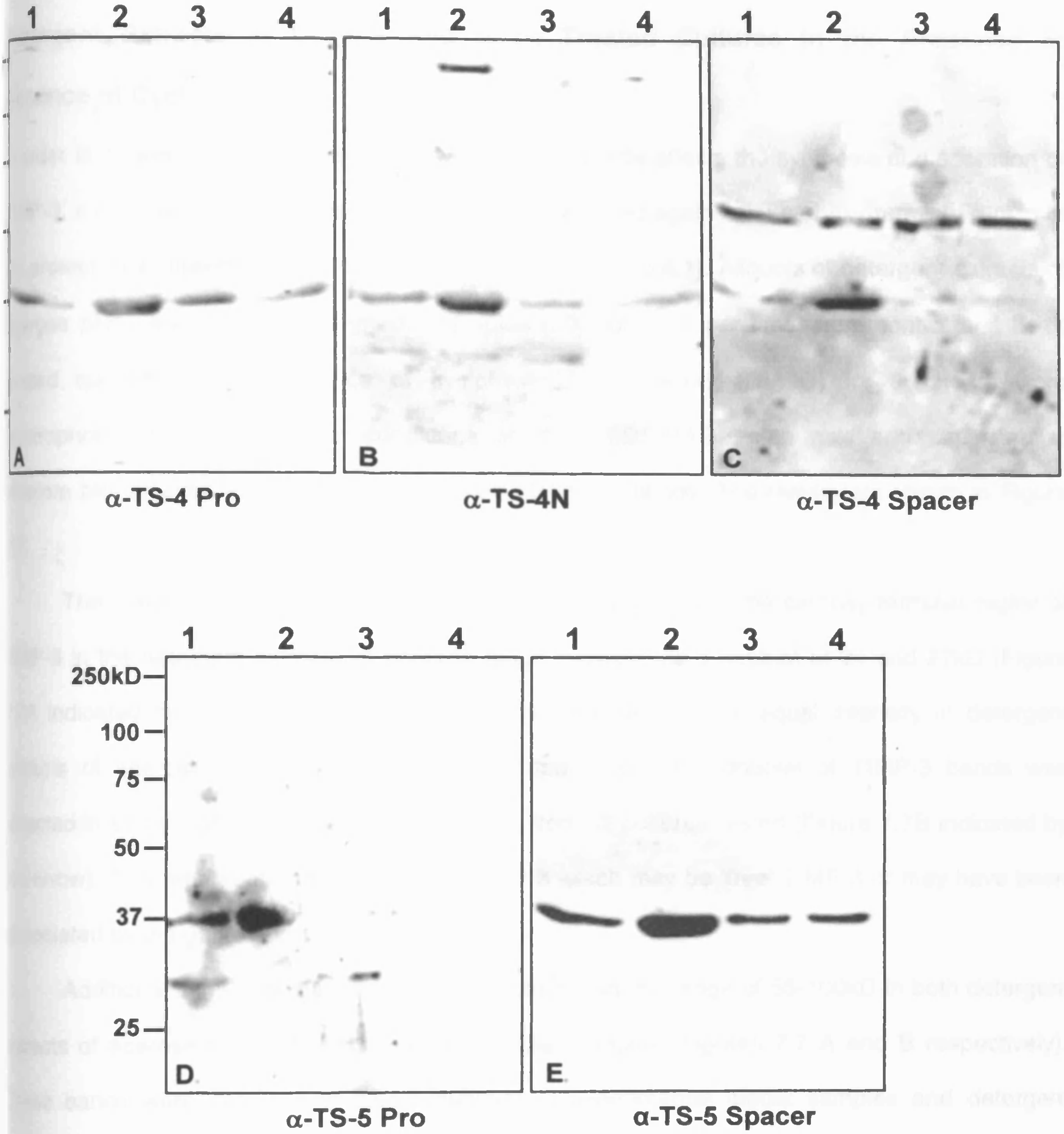


Figure 7.6 Western blot analyses of ADAMTS-4 and –5 isoforms bound to a Heparin-Sepharose column and eluted in 0.8M sodium chloride (50μl per lane) from media samples treated for 96 hours with (1) Control + DMSO, (2) IL-1α (10ng/ml) + DMSO, (3) Control + cycloheximide (CHX [5μg/ml]) and (4) IL-1α (10ng/ml) + cycloheximide (CHX [5μg/ml]). Western blots were probed with antibodies to (A) The amino-terminal prodomain of ADAMTS-4 (α-TS-4 Pro), (B) The amino-terminal end of the metalloproteinase domain of ADAMTS-4 (α-TS-4N), (C) The carboxy-terminal spacer domain of ADAMTS-4 (α-TS-4 Spacer), (D) The amino-terminal prodomain of ADAMTS-5 (α-TS-5 Pro) and (E) The spacer domain of ADAMTS-5 (α-TS-5 Spacer).

7.4.5 Western Blot Analysis of TIMP-3 Present in the Experimental Medium and Detergent Extracts of Control and IL-1α Treated Cultures in the Presence or Absence of Cycloheximide

In order to determine whether treatment with cycloheximide affects the synthesis and secretion of TIMP-3, a commercially available polyclonal antibody raised against the carboxy-terminal region of the protein was utilised (as described in Chapter 6 Section 6.4.1). Aliquots of detergent extracts of agarose plugs and experimental media samples (50μl of each per lane) from control and IL-1α treated cultures in the presence of cycloheximide or carrier (DMSO) for 96 hours were electrophoresed under reducing conditions on 12% SDS-PAGE slab gels and subjected to Western blot analysis using the polyclonal Anti-TIMP-3 antibody. The results are shown in Figure 7.7.

The major immunopositive band detected by the antibody to the carboxy-terminal region of TIMP-3 in the detergent extracts of agarose plugs migrated as a doublet at 24 and 27kD (Figure 7.7A indicated by red arrow). These two bands were detected in equal intensity in detergent extracts of agarose plugs from all cultures tested. The same doublet of TIMP-3 bands was detected in equal intensity in the media samples from all cultures tested (Figure 7.7B indicated by red arrow). This represents non-associated TIMP-3 which may be 'free' TIMP-3 or may have been dissociated by the gel running conditions.

Additional immunopositive bands were detected in the range of 55-100kD in both detergent extracts of agarose plugs and experimental media samples (Figures 7.7 A and B respectively). These bands were detected in equal intensity in experimental media samples and detergent extracts of agarose plugs from all cultures tested. A low molecular weight immunopositive band was detected at 17kD in media samples from agarose cultures treated in the presence of cycloheximide. This band was absent from media samples from cultures treated in the absence of cycloheximide (Figure 7.7B).

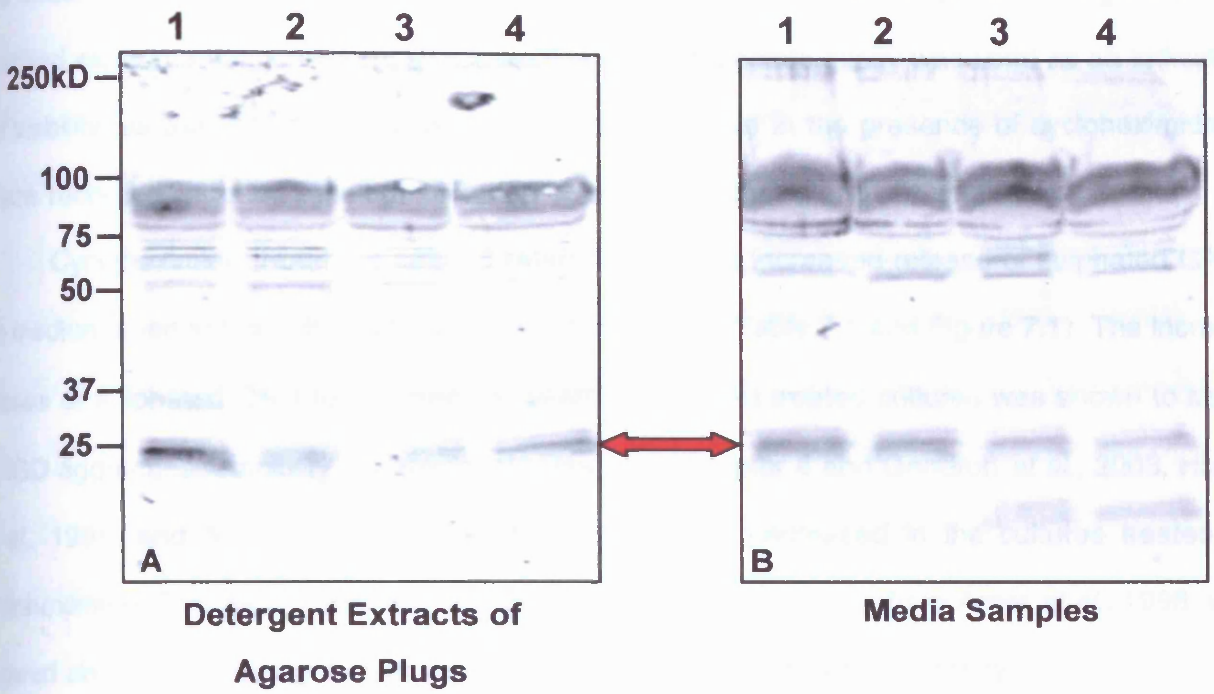


Figure 7.7 Western blot analysis of TIMP-3 present in aliquots of (A) Detergent extracts of agarose plugs and (B) Media samples (50μl per lane) from cultures treated for 96 hours with (1) Control + DMSO, (2) IL-1α (10ng/ml) + DMSO, (3) Control + cycloheximide (CHX [5μg/ml]) and (4) IL-1α (10ng/ml) + cycloheximide (CHX [5μg/ml]). Western blots were probed with a commercially available polyclonal antibody raised against the carboxy-terminal region of TIMP-3.

7.5 Discussion

The effect of cycloheximide on the metabolism of the chondrocytes embedded in agarose under specified experimental conditions was investigated. The Lactate assay was used as an indicator of cell viability, as the chondrocytes were metabolically active in the presence of cycloheximide any effects recorded were the result of inhibition of protein synthesis and not apoptosis.

Cycloheximide treatment caused retardation of the increased release of sulphated GAG to the medium seen in the cultures treated with IL-1α alone (Table 7.1 and Figure 7.1). The increased release of sulphated GAG to the medium seen in the IL-1α treated cultures was shown to be due to 'IGD aggrecanase activity' as previously reported (Chapter 4 and Gendron *et al.,* 2003, Hughes *et al.,* 1995, and Arner *et al.,* 1998). This activity was decreased in the cultures treated with cycloheximide. This data correlates with previously published results from Arner *et al.,* 1998, which showed *de novo* protein synthesis to be required for 'IGD aggrecanase activity'.

Isoforms of ADAMTS-4 and –5 were detected in equal amounts in zinc chelator bound fractions from media samples from all experimental culture conditions tested (Figures 7.5 and 7.4, respectively). In detergent extracts of agarose plugs high molecular weight isoforms of ADAMTS-4 and -5 were detected in apparent equal intensity in all treatments tested. However, the lower molecular weight isoforms of ADAMTS-4 and -5 were greatly decreased in intensity in cycloheximide treated cultures (both control and IL-1α treated) (Figures 7.4 A-E Lanes 3 and 4) compared to cultures treated with carrier (DMSO) (Figures 7.4 A-E Lanes 1 and 2). These results indicate that *de novo* protein synthesis in serum free conditions must be required for generation of these low molecular weight isoforms. The small isoforms may result from alternative splicing occurring in serum free conditions which were not generated due to the presence of cycloheximide. Alternatively, another protease with a rapid turnover may be responsible for generation of these small isoforms by catalysis of higher molecular weight forms of ADAMTS-4 and -5. Each of the isoforms of ADAMTS-4 and –5 present in zinc chelator bound fractions of media samples and detergent extracts of agarose plugs are discussed in detail in Chapter 5 Section 5.5.

As previously reported in this thesis the predominant heparin bound isoforms of ADAMTS-4 and –5 co-migrate at 37kD (Figure 7.6) with an additional 55kD isoform of ADAMTS-4 being detected by the antibody to the spacer domain of the protein (Figure 7.6C). The co-migrating 37kD isoforms of ADAMTS-4 and -5 were detected at increased prevalence in IL-1α treated cultures compared to controls. The addition of the cycloheximide carrier (DMSO) had no effect on this banding pattern (Figures 7.6 A-C Lanes 1 and 2). Interestingly, in the cultures treated with cycloheximide in the presence of IL-1α there was no increase in the 37kD isoforms of ADAMTS-4 and -5 (Figures 7.6 A-E Lane 4) compared to the levels detected in cultures treated with cycloheximide alone (Figures 7.6 A-E Lane 2). It has recently been reported that carboxy-terminal truncation enhances the 'IGD aggrecanase activity' of ADAMTS-4 (Gao *et al.*, 2002), thus implying a role for the carboxy-terminally truncated 37kD isoforms of ADAMTS-4 and –5 in the increased 'aggrecanase activity', detected at the site within the IGD, in the presence of IL-1α (Figure 7.3C). This is also suggested by the fact that the co-migrating 37kD immunopositive bands of ADAMTS-4 and –5 are detected in equal intensity in the cultures treated with cycloheximide in the presence or absence of IL-1α. Alternatively, the enzyme responsible for catabolism of ADAMTS-4 and –5 resulting in the 37kD isoform may be rapidly turned over and therefore swiftly lost from cultures where *de novo* protein synthesis has been inhibited. For example MT4-MMP has been shown to cleave ADAMTS-4 resulting in truncated isoforms able to cleave aggrecan at the Glu^{373}-Ala^{374} bond within the interglobular domain in a chondrosarcoma cell line (Gao *et al.*, 2004).

The major TIMP-3 immunopositive bands detected in both detergent extracts of agarose plugs and media samples showed no major differences between any of the experimental culture conditions tested (Figure 7.7). Immunopositive bands containing TIMP-3 associated with other matrix proteins were detected at 55, 60, 70, 75 and 100kD. The ability of TIMP-3 to interact with constituents of the extracellular matrix is unique among members of the TIMP family (Pavloff *et al.*, 1992 and Yu *et al.*, 2000). TIMP-3 bands were also detected at 24 and 27kD either corresponding to 'free' TIMP-3 or TIMP-3 dissociated under reducing conditions. Additional TIMP-3 immunopositive bands were detected at 17kD in media samples from cultures treated with cycloheximide which were absent from cultures treated with carrier (DMSO). This may correspond to a degraded form of TIMP-3 which is removed by a protease with a rapid turnover. Therefore in the presence of cycloheximide, synthesis of the degrading protease is inhibited and as a result partially degraded forms of TIMP-3 are detectable. ADAMTS-4 has recently been shown to cleave

TIMP-4, but not TIMPs-1, 2 and -3 at the A^{192}-Q^{193} site and Neutrophil elastase has been shown to cleave TIMP-1 at the Val^{69}-Cys^{70} bond (Zang *et al.,* 2004). However, no such activity has yet been described against TIMP-3.

7.6 Summary

- *De novo* protein synthesis in serum free conditions is required for 'aggrecanase activity' within the interglobular domain of aggrecan.
- *De novo* protein synthesis is also required for the generation of co-migrating heparin binding 37kD isoforms of ADAMTS-4 and –5, which in the absence of cycloheximide are increased in cultures treated with IL-1α compared to untreated controls. These isoforms may result from enzyme autocatalysis, alternative splicing or catalytic processing by another enzyme such as MT4-MMP (Gao *et al.,* 2004).

8 General Discussion

In this investigation the model culture system of chondrocytes embedded in agarose was used to study the degradation of aggrecan by ADAMTS-4 and -5, and the synthesis, sequestration and activation these aggrecanases in cartilaginous extracellular matrices. Chondrocyte-agarose cultures secrete an extracellular matrix rich in aggrecan during the preculture period in the presence of serum. The aggrecan was degraded in serum free conditions resulting in the release of sulphated GAG to the culture medium. The proportion of the total GAG present released to the medium during the treatment time was substantially increased by the presence of IL-1α. In chondrocyte-agarose cultures treated with IL-1α >70% of the total GAG present was released to the medium in just 24 hours, in contrast to achieve release of >70% of the total GAG present from bovine nasal cartilage explant cultures treatment with IL-1 for 7 days was required (Little *et al.*, 2002b) similarly only 65% of the total GAG present was released from bovine articular cartilage explant cultures treated with IL-1 in 15 days (Sandy *et al.*, 1991a). Thus the model system of chondrocytes embedded in agarose is extremely useful for the rapid analysis of the effects of pharmaceutical agents on the metabolism of chondrocytes. Further analysis of chondrocyte-agarose cultures as a model system should be carried out. This may include analysis of mRNA and protein levels for various matrix macromolecules, including types II and VI collagen, aggrecan and other proteoglycans, as well as matrix proteases, such as MMPs, ADAMTSs and cathepsins, and inhibitors such as TIMPs.

Aggrecan degradation detected as sulphated GAG release to the culture medium was found to be due to cleavage at the 'aggrecanase site' within the IGD of the aggrecan core protein. No MMP activity against the IGD of aggrecan was detected. This finding corresponds with numerous previously published results, which detected increased aggrecanase-generated aggrecan catabolites in the medium of explant cultures treated with IL-1 (Gendron *et al.*, 2003, Hughes *et al.*, 1995, and *Arner et al.*, 1998).

The focus of this investigation was aggrecan degradation, and within the culture period used the major cause of aggrecan degradation was due to 'IGD aggrecanase activity'. Since the activities of ADAMTS-4 and -5 against the 'aggrecanase site' within the IGD of aggrecan are indistinguishable, and they are thought to be key players in the degradation of aggrecan in diseases such as osteoarthritis and rheumatoid arthritis (Sandy *et al.,* 1992, and Lohmander *et al.,* 1993), their secretion, sequestration and activation in this model system was investigated using a range of mono- and polyclonal antibodies that recognise different domains of ADAMTS-4 and -5.

Numerous immunopositive bands were detected with antibodies to ADAMTS-4 and -5 in both detergent extracts of agarose plugs and media samples indicating multiple isoforms of the enzymes to be present in this culture system. Detergent extracts of agarose plugs may contain proteins which are membrane-bound or associated as well as intracellularly located or sequestered within the extracellular matrix. Media samples were partially purified via passage over a Heparin-Sepharose column followed by a Zinc-Chelator column.

The high molecular weight ADAMTS-4 and -5 isoforms detected in detergent extracts of agarose plugs and zinc chelator bound media fractions were unlikely to be involved in the massive increase in 'IGD aggrecanase activity' detected in IL-1α treated cultures compared to controls since they appeared not to be upregulated by IL-1α treatment. The enzyme isoforms detected may be inactive, due to them possessing a prodomain, or catalytically active due to removal of their prodomain by Furin (Molloy *et al.,* 1992, Gao *et al.,* 2002, Tortorella *et al.,* 1999, Abbaszade *et al.,* 1999, and Wang *et al.,* 2004) but inactivated by sequestration within the matrix or the presence of proposed endogenous physiological inhibitors such as TIMP-3 (Kashiwagi *et al.,* 2001) or α_2 Macroglobulin (Tortorella *et al.,* 2004). Furthermore the Furin-cleaved intact form of recombinant human ADAMTS-4 has been shown to preferentially cleave aggrecan at the KEEE^{1667}-^{1668}GLGS and GELE^{1480}-^{1481}GRGT sites within the carboxy-terminal GAG binding region rather than the NITEGE^{373}-^{374}ARGSV ('aggrecanase site') within the IGD of aggrecan (Tortorella *et al.,* 2000b).

Recently published data showed a recombinant form of human ADAMTS-4 to bind to the cell surface and extracellular matrix of the cells in which it was expressed (293-EBNA cells) (Kashiwagi *et al.,* 2004). This interaction was dissociated by addition of heparin allowing release of the recombinant enzyme into the culture medium. Furthermore, Pratta *et al.,* 2003 reported addition of heparin to IL-1 treated bovine monolayers inhibited 'IGD aggrecanase activity'. Therefore, it could be concluded that the active form of aggrecanase must have the ability to bind heparin. Thus implicating the isoforms of ADAMTS-4 and -5 bound by the Heparin-Sepharose column as complicit in the 'IGD aggrecanase activity' seen in the presence of IL-1α in the culture system of chondrocytes embedded in agarose, and potentially as the active aggrecanase(s) in cartilage degradation in arthritis.

The heparin bound ADAMTS-4 and -5 isoforms detected co-migrated at ~37kD and were present in apparently increased amounts with increasing time in serum free medium and in IL-1α treated cultures compared to controls. This is similar to previously published results indicating treatment with IL-1β to increase the prevalence of lower molecular weight isoforms of ADAMTS-4 in cell lysates from bovine chondrocyte monolayers (Pratta *et al.,* 2003).

Generation of apparently increased levels of co-migrating 37kD isoforms of ADAMTS-4 and -5 in IL-1α treated cultures was inhibited by cycloheximide, and therefore required *de novo* protein synthesis in the presence of IL-1α for their generation. As previously reported, *de novo* protein synthesis was also required for 'IGD aggrecanase activity' detected in the presence of IL-1 (Arner *et al.,* 1998). This further implicates the co-migrating 37kD isoforms of ADAMTS-4 and -5 to be involved in the 'IGD aggrecanase activity' detected in IL-1 treated cultures.

The co-migrating 37kD isoforms of ADAMTS-4 and -5 may result from enzyme catalysis by autocatalysis due to a lack of suitable substrate. Chondrocyte-agarose cultures treated in the presence of IL-1α release over 70% of the sulphated GAG present to the medium in 24 hours (see Chapter 4). Therefore following 24 hours of treatment in the presence of IL-1α 70% of the substrate for ADAMTS-4 and –5 has been lost to the medium consequently by 48 hours of treatment in the presence of IL-1α the enzymes themselves may be undergoing autocatalysis. Alternatively, the 37kD isoforms of ADAMTS-4 and –5 may result from alternative splicing or catalytic activation by another enzyme. Since these 37kD isoforms of ADAMTS-4 and –5 are increased in IL-1α treated cultures compared to controls they may play a role in the increased 'IGD aggrecanase activity' detected in these cultures (see Chapter 4).

The possibility of alternatively spliced forms of ADAMTS-4 and -5 could be investigated by PCR analysis using primers recognising sequences located in various areas of the enzymes. This may allow elucidation of which domains of ADAMTS-4 and -5 are expressed in the presence or absence of IL-1α.

A model for the generation of 55 and 40kD isoforms of ADAMTS-4 was recently described in which catalysis occurs at the cell surface via membrane bound MT4-MMP (Gao *et al.*, 2004) In this model cleavage occurs at the Lys^{694}-Phe^{695} bond within the cysteine-rich domain resulting in a ~55kD isoform and at the Thr^{581}-Phe^{582} bond within the thrombospondin-1 like domain resulting in a ~40kD isoform. The 40kD isoform described using human peptide sequence by Gao *et al.*, 2004 may have a molecular weight of ~37kD in porcine chondrocytes. A similar mechanism may be proposed for generation of 37kD isoforms of ADAMTS-5. To further investigate this possibility MMP specific inhibitors, such as TIMP-1 or -2, may be utilized. Inhibition of 'IGD aggrecanase activity' by the presence of such inhibitors would indicate MMP activity to be necessary for activation of 'aggrecanase'.

Previously published data suggested lower molecular weight isoforms of ADAMTS-4 and -5 to result from truncation of the proteins resulting in loss of the carboxy-terminal regulatory regions of the enzymes (Pratta *et al.*, 2003, Kashiwagi *et al.*, 2004, Gao *et al.*, 2002, Gao *et al.*, 2004 and Flannery *et al.*, 2002). Interestingly, full length Furin cleaved recombinant human ADAMTS-4 showed little catalytic activity against the 'aggrecanase site' ($NITEGE^{373}$-$^{374}ARGSV$) within the IGD of aggrecan, preferentially cleaving the $GELE^{1480}$-$^{1481}GRGT$ site within the carboxy-terminal GAG binding region (Tortorella *et al.*, 2000b, and Kashiwagi *et al.*, 2004). Whereas truncated forms of ADAMTS-4 lacking the carboxy-terminal spacer domain showed apparently increased catalytic activity to the $NITEGE^{373}$-$^{374}ARGSV$ ('aggrecanase site') within the IGD of the aggrecan core protein as well as increased non-specific catalytic activity to other matrix proteins such as decorin and fibromodulin (Kashiwagi *et al.*, 2004). This implies that generation of lower molecular weight isoforms of ADAMTS-4 and -5 may either constitute 'activation' of these enzymes as predicted by Pratta *et al.*, 2003, or it may represent a deregulation of the enzymes leading to more promiscuous catalytic activity (Gao *et al.*, 2002, Gao *et al.*, 2004, and Kashiwagi *et al.*, 2004). Therefore the high molecular weight enzyme isoforms may be required to play normal physiological roles, whereas the low molecular weight forms are likely to be the enzyme isoforms involved in the destruction of aggrecan and other proteoglycans in articular cartilage during arthritis. This suggests targeting of enzyme inhibitors against the active sites of ADAMTS-4 and -5 to be inopportune as such inhibitors would not distinguish between high and low molecular weight forms of the enzymes and may therefore disrupt the physiological role played by the high molecular weight isoforms of the proteins.

Media fractions from IL-1α treated cultures were found to possess soluble 'aggrecanase activity' against the IGD of exogenously added aggrecan (A1D1) which was absent from media samples from control cultures. The heparin bound media fractions from IL-1α treated cultures consistently contained apparently high levels of 'IGD aggrecanase activity', whereas in contrast the activity of the zinc bound media fractions against the IGD of exogenous aggrecan was extremely variable. This may suggest the 'IGD aggrecanase activity' detected in the zinc bound media fractions to be due to contamination with low molecular weight heparin binding catalytically active isoforms of ADAMTS-4 and / or 5. Alternatively, catalysis of the high molecular weight zinc bound isoforms may occur to form lower molecular weight catalytically active isoforms of the enzymes (Flannery *et al.*, 2002, and Gao *et al.*, 2004).

The 'IGD aggrecanase activity' detected in heparin bound media fractions from IL-1α treated cultures was ablated by addition of monoclonal antibody Anti-TS-4N to the digestion mix. This indicates the 'IGD aggrecanase activity' detected in these cultures to be predominantly due to isoforms of ADAMTS-4 rather than ADAMTS-5. Furthermore, monoclonal antibody Anti-TS-4N detected a single band at 37kD by Western blot analysis implying a low molecular weight ADAMTS-4 isoform to be responsible for the detected 'aggrecanase activity'. This is similar to previously published results which show immunodepletion of media taken from IL-1α stimulated cartilage using an ADAMTS-4 polyclonal antibody led to a 75% reduction in 'IGD aggrecanase activity', whilst immunodepletion with an ADAMTS-5 antibody led to only a 15% decrease in 'IGD aggrecanase activity' (Tortorella *et al.,* 2001). These results suggest that an antibody affinity column could be produced, using monoclonal antibody Anti-TS-4N, and used to purify isoforms of ADAMTS-4 possessing 'IGD aggrecanase activity'.

The 'aggrecanase activity' of both the heparin and zinc bound media fractions, from IL-1α treated cultures, against the IGD of exogenous aggrecan was inhibited by the presence of TIMP-3 or a recombinant protein comprising the amino-terminal domain of TIMP-3 (N-TIMP-3). Therefore the enzyme(s) possessing 'IGD aggrecanase activity' present in the heparin or zinc bound media fractions from, IL-1α treated cultures, were inhibited by the amino-terminal region of TIMP-3. ADAMTS-4 and -5 have previously been shown to be inhibited by the amino-terminal region of TIMP-3 leading to its nomination as a physiological inhibitor of these enzymes *in vivo* (Kashiwagi *et al.*, 2001).

In order to determine whether TIMP-3 is present in the model system of chondrocytes embedded in agarose an antibody to the carboxy-terminal region of the protein was used. Endogenous TIMP-3 was detected in this culture system as low molecular weight 'free' TIMP-3 (i.e. TIMP-3 not associated with other molecules) and high molecular weight bands of TIMP-3 associated with matrix components or enzymes. This ability of TIMP-3 to bind to extracellular matrix components is unique among members of the TIMP family (Pavloff *et al.*, 1992, and Yu *et al.*, 2000) and may be further investigated using the TIMP-3 antibody to co-immunoprecipitate the TIMP-3 and any associated macromolecules. No differences were apparent in the total level of TIMP-3 protein present between control and IL-1α treated cultures. Interestingly, lower levels of 'free' TIMP-3 were detected in IL-1α treated cultures compared to controls, possibly indicating higher levels of bound TIMP-3 in these cultures. Overall, TIMP-3 synthesis was not upregulated by treatment with IL-1α. Therefore TIMP-3 synthesis may not be involved in the compensation mechanisms employed by chondrocytes in this model system when exposed to catabolic stimulants such as IL-1α. Levels of TIMP-3 detected were also unaffected by treatment with cycloheximide, indicating *de novo* protein synthesis following treatment in serum free conditions to be unnecessary for generation of the TIMP-3 protein detected.

The amino-terminal region of TIMP-3 is thought to be a physiological inhibitor of ADAMTS-4 and -5 (Kashiwagi *et al.*, 2001) and has been shown to be a potent inhibitor of 'IGD aggrecanase activity' in the culture system of chondrocytes embedded in agarose when added exogenously. TIMP-3 protein has also been detected endogenously in this culture system. Therefore the ability of the ADAMTS-4 and -5 isoforms previously detected in this model system to bind to the amino-terminal region of TIMP-3 was investigated using a recombinant protein comprising the amino-terminal domain of TIMP-3 (N-TIMP-3). The ADAMTS-4 and -5 able to bind to N-TIMP-3, when used as a purification column, were only those enzyme isoforms predicted to be intact apart from cleavage at the Furin site. Interestingly, none of the low molecular weight 37kD isoforms of ADAMTS-4 or 5 were able to bind to the N-TIMP-3. This is in contrast to previously published work using binding to N-TIMP-3 as a purification method for ADAMTS-4 isoforms. Here multiple isoforms of ADAMTS-4 were detected bound by N-TIMP-3, these ranged in molecular weight from 37-45kD (Kashiwagi *et al.,* 2004). In the culture system of chondrocytes embedded in agarose the binding of ADAMTS-4 and -5 to N-TIMP-3 may be hindered by the presence of endogenous TIMP-3 rather than the lack of an appropriate site of interaction on the enzyme isoform. In the published study samples were incubated with N-TIMP-3 under dissociative conditions allowing for no such interaction between endogenous TIMP-3 and ADAMTS-4 (Kashiwagi *et al.,* 2004), although it is possible that such conditions may also have interfered with binding of ADAMTS-4 isoforms to N-TIMP-3. None the less it is interesting to speculate why addition of exogenous N-TIMP-3 inhibited the 'IGD aggrecanase activity' of media samples from IL-1α treated cultures whilst N-TIMP-3 was unable to bind the ADAMTS-4 isoforms thought to be responsible for this 'IGD aggrecanase activity' (i.e. the low molecular weight 37kD ADAMTS-4 isoform detected by M'Ab Anti-TS-4N).

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